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Canonical and non-canonical regulation of AMP-activated protein kinase

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Abbreviations

4E-BP	Eukaryotic translation initiation factor 4E-binding protein
ACC	Acetyl Co-A carboxylase
AICAR	5-amino-imidazole carboxamide riboside
AID	Autoinhibitory domain
AMPK	AMP-activated protein kinase
AU	Arbitrary units
Bis	Bis-acrylamide
BSA	Bovine serum albumin
CaMKK	Ca^{2+}/calmodulin-dependent protein kinase kinase
CBM	Carbohydrate binding module
CBS	Cystathione-b-synthase
CPT1	Carnitine:palimitoyl-CoA transferase 1
CRTC2	CREB-regulated transcription co-activator 2
CTD	C-terminal domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic initiation factor
ePK	Eukaryotic protein kinase
FBS	Foetal bovine serum
FLAG	DYDDDDK peptide
FOXO	Forkhead box O
FRT	Flp-recombinase target
GAP	GTP-ase activating protein

GBD	Glycogen binding domain
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GLUT	Glucose transporter
GST	Glutathione-S-transferase
GS	Glycogen synthase
HDAC	Histone deacetylase
HEK-293	Human embryonic kidney 293 cells
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HMGR	3-hydroxy-3-methyl-glutaryl-CoA reductase
IGF	Insulin-like growth factor
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRS	Insulin receptor substrate
KD	Kinase domain
LB	Luria-Bertrani broth
LKB1	Liver kinase B1
M	Molar
MEF	Mouse embryonic fibroblast
MO25	Mouse protein 25
MOPS	3-(n-morpholino) propane sulfonic acid
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
OCR	Oxygen consumption rate
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase chain reaction
PDK	3-phosphoinositide-dependent protein kinase 1
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
PGC1α	Peroxisome proliferator-activated recptor gamma coactivator 1-α

PI3K	Phosphatidylinosited 3-kinase
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP2	Phosphatidylinositol (3,4,5)-trisphosphate
PJS	Peutz-Jeghers syndrome
PKA	Cyclic AMP-dependent protein kinase
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethylsulfonylfluoride
PP	Protein phosphatase
PTEN	Phosphatase and tensin homologue
Rab	Ras-related in brain
Raptor	Regulatory associated protein of mTOR
Rheb	Ras homologue enriched in brain
Rictor	Rapamycin insensitive companion of mTOR
RNA	Ribonucleic acid
SBTI	Soya bean trypsin inhibitor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SREBP	Sterol regulatory element-binding protein
STRAD	Ste20-related protein
TBS	Tris buffered saline
TOR	Target of rapamycin
Tris	Tris(hydroxymethyl) methylamine
TSC	Tuberous sclerosis complex
ULK1	Unc-51-like kinase 1
WPW	Wolff-Parkinson-White
WT	Wild type
ZMP	5-aminoimidazole-4-carboxamide ribonucleoside monophosphate
α–RIM	α regulatory-subunit-interacting motif

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Declaration

I hereby declare that the following thesis is based on results of investigations conducted by myself, and that this thesis is my own composition. Work other than my own is clearly indicated in the text. This dissertation has not in whole, or in part, been previously presented for higher degree.

Francesca Romana Auciello

I certify that Francesca Romana Auciello has spent the equivalent of at least nine terms in research work in Division of Cell Signalling and Immunology, University of Dundee, and that she has fulfilled the conditions of the Ordinance No 39 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Prof. D. Grahame Hardie

Summary

The AMP-activated protein kinase (AMPK) is a sensor of cellular energy stress that, once activated, promotes ATP-producing process while it switches off ATP-consuming pathways, in order to restore the cellular energetic balance under conditions of stress. Activation of AMPK is dependent on the phosphorylation of the residue Thr172 in its α subunit. This phosphorylation is generally mediated by the known tumour suppressor LKB1, but also CaMKKβ has been shown to phosphorylate AMPK. As its name suggests, AMPK is also activated by the binding of AMP to its γ subunit. This binding causes a >10 fold allosteric stimulation, promotes phosphorylation of Thr172 by upstream kinases and protects AMPK from dephosphorylation of Thr172 by protein phosphatase(s).

In 2010 it was reported that oxidative stress mediated by H_2O_2 activated AMPK by increasing the cellular AMP:ATP and ADP:ATP ratios (Hawley et al, 2010). However, the same year another work suggested that the mechanism of activation of AMPK by H_2O_2 was direct, independent of AMP and involved the oxidation of two cysteine residues in the α subunit of AMPK (Zmijewski et al, 2010). Given this discrepancy, here we provided evidence that H_2O_2, generated by addition of glucose oxidase in the cell medium, activates AMPK mostly through an increase of AMP:ATP and ADP:ATP ratios, as previously suggested in our laboratory. However, it seems that there might be a second, minor mechanism of activation that is independent of the changes in cellular nucleotides. This second mechanism was not identified in our previous work because we were not aware of how rapidly a single bolus of H_2O_2 can be metabolized by the antioxidant defences of the cell. We could not identify the alternative mechanism of activation by H_2O_2 but showed that H_2O_2 could protect Thr172 from dephosphorylation, which might suggest a direct effect of H_2O_2 on the phosphatase(s)

dephosphorylating AMPK. However, since the identity of this phosphatase(s) remains unclear, we could not rule out the possibility that the protection from dephosphorylation that we observed could still be mediated by the increase in AMP:ATP and ADP:ATP ratios. Moreover, it remains still possible that a direct effect of H_2O_2 on AMPK might be responsible for the small but significant activation we detected in cell expressing a nucleotides-insensitive mutant of AMPK.

Recently, a new crystal structure of AMPK obtained by Xiao et al (2013) provided new insights about AMPK structure and regulation. In particular, the authors identified a new binding pocket located at the interface between the N-lobe of the α-kinase domain and the β-CBM of AMPK, which appeared to be the binding site for two direct activators of AMPK: A769662 and 991. Here we confirm that this novel binding pocket is indeed the binding site for both A769662 and 991, and provide evidence that another direct activator of AMPK, MT63-78, also binds at the same site. Mutation of two important residues in this pocket (Lys29 and Lys31 of the α2 subunit) abolished the allosteric stimulation of AMPK by A769662, 991 and MT63-78 while it had no effect on allosteric stimulation by AMP. However, we also showed that the same mutation abolished protection against Thr172 dephosphorylation not only by A769662, 991 and MT63-78, but also by phenformin and H_2O_2, which are known to activate AMPK by increasing the AMP:ATP and ADP:ATP ratios. These data show that the integrity of this pocket is important for the effect of AMP to protect against Thr172 dephosphorylation, but not for its ability to cause allosteric stimulation. Moreover, in HEK-293 cell stably expressing an α2 subunit carrying the mutation of both Lys29 and Lys31, the basal activity of AMPK due to Thr172 phosphorylation was almost 6-fold less than in cells expressing wild-type α2. This result pointed out for the first time that

there might be a natural ligand binding in the newly discovered binding pocket that is not able to bind to the double mutant, explaining the difference in activity observed. However the identity of this possible natural ligand remains unclear and more studies will be necessary to uncover it.

Publication

Some of the results in this thesis have been presented in the following publication:

Auciello, F.R., Ross, F.A., Ikematsu, N. and Hardie, D.G., 2014. Oxidative stress activates AMPK in cultured cells primarily by increasing cellular AMP and/or ADP. FEBS Letters 18, 3361-6.

Chapter 1: Introduction

1.1 Protein phosphorylation

Protein phosphorylation is a post translational modification involving the transfer of a phosphate group from ATP to the hydroxyl group of a serine, threonine or tyrosine residue of a protein. The enzymes catalysing phosphorylation are called protein kinases while those reversing this reaction, removing the phosphate group, are known as protein phosphatases. The reversible phosphorylation of proteins regulates nearly every aspect of cell life (Cohen, 2002). Phosphorylation alters the structural conformation of a protein, resulting in the modification of its function by several different ways: i) by increasing or decreasing its activity; ii) by changing its subcellular localization; iii) by promoting or preventing interactions with other proteins; iv) by increasing its stability or labelling it for destruction. Protein phosphorylation is involved in a variety of signalling transduction pathways and its abnormal regulation is now recognised as a common event in many human diseases. The first reaction recognized to be regulated by protein phosphorylation was the conversion of inactive glycogen phosphorylase *b* to active glycogen phosphorylase *a*, achieved in the presence of Mg-ATP and catalysed by an enzyme called phosphorylase kinase (Fischer & Krebs, 1955). The concept of a signalling cascade was first defined in 1968 when cyclic-AMP dependent protein kinase (PKA) was shown to phosphorylate and inactivate phosphorylase kinase (Walsh et al, 1968). Sequencing the human genome allowed the identification of 518 putative protein kinases, of which 478 contain the classical “eukaryotic protein kinase” (ePK) catalytic kinase domain (Manning et al, 2002). Protein kinases are grouped into families based on the sequence similarities of

their catalytic domains, the domain structure outside the catalytic domain and the known biological functions (Manning et al, 2002).

1.2 AMP-activated protein kinase (AMPK): discovery and historical background

3-Hydroxyl-3-methylglutaryl coenzyme A reductase (HMGR) converts HMG-CoA to mevalonic acid, playing a crucial role in the cholesterol biosynthesis pathway. Beg et al (Beg et al, 1973) described a reduction in the activity of HMGR that was dependent upon a cytosolic fraction and MgATP. HMGR could then be reactivated by incubation with another cytosolic fraction, demonstrating that HMGR activity could be interconverted between active and inactive states. Nordstrom et al (Nordstrom et al, 1977) showed that the inactivation of HMGR could be reversed by an activating factor that could be completely inhibited by the protein phosphatase inhibitor sodium fluoride, suggesting that regulation of HMGR activity relied on changes in its phosphorylation status. More conclusive evidence came from the incubation of HMGR with Mg^{2+} ions and [γ-^{32}P]ATP, which resulted in increased ^{32}P-incorporation into the enzyme and a decrease in its activity. Conversely, incubation with partially purified phosphatase released ^{32}P and increased enzyme activity (Beg et al, 1978). It was thus clear that HMGR activity was regulated by a cycle of phosphorylation and dephosphorylation. In 1985, Ferrer and colleagues demonstrated that 5'-AMP bound and activated the HMGR kinase (Ferrer et al, 1985). Concurrently with the above studies, Carlson and Kim (1973) showed that incubation with MgATP inactivated acetyl-CoA carboxylase (ACC), an enzyme involved in long-chain fatty acid synthesis. The reduced activity occurred along with the incorporation of ^{32}P into protein and was dependent on the presence of a protein fraction presumed to contain an ACC kinase.

Also, the reactivation of ACC, following incubation with $MgCl_2$, was sensitive to sodium fluoride, suggesting the presence of a phosphatase and, consequently, that ACC activity is also regulated by a cycle of phosphorylation and dephosphorylation. It was later reported that 5'-AMP promoted the inactivation of ACC, although this was incorrectly attributed to a direct effect of AMP on ACC itself (Yeh et al, 1980). Despite this, the mechanism of regulation of ACC clearly resembled that seen with HMGR, and in 1989 Carling and co-workers obtained evidence to show that it was the same kinase inactivating both ACC and HMGR, through an AMP-stimulated mechanism (Carling et al, 1989). The unifying name of AMP-activated protein kinase (AMPK) firstly appeared in the literature in 1988 (Munday et al, 1988).

1.3 AMPK structure: the α, β and the γ subunits

AMPK exists throughout eukaryotes as a heterotrimeric complex composed of a catalytic α subunit and regulatory β and γ subunits. In mammals each subunit is encoded by distinct genes and is present as multiple isoforms (α1, α2; β1, β2; γ1, γ2, γ3) (Grahame Hardie, 2014). The α subunit was the first to be identified, showing a molecular mass of 63 kDa (Carling et al, 1989) while two additional polypeptides of 38 and 35 kDa were subsequently purified (Davies et al, 1994). These two polypeptides are now known as β and γ subunits and, along with the α subunit were shown to co-migrate through gel filtration and to be present in a molar ratio of 1:1:1 (Davies et al, 1994; Mitchelhill et al, 1994). Figure 1.1 shows a schematic representation of AMPK subunits.

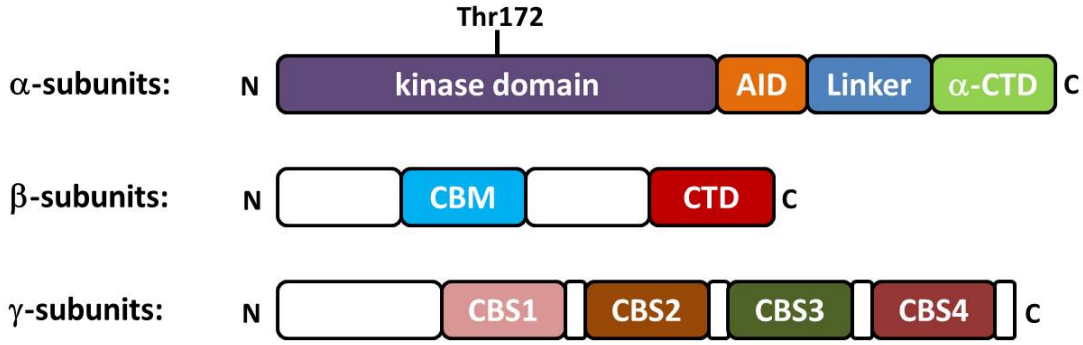

Figure 1.1. The α,β and γ subunits of AMPK. Linear layout of the domains forming a typical mammalian AMPK heterotrimer.

1.3.1 The α subunit

The first α subunit identified was the one that we now know as α2 (Stapleton et al, 1996). It showed a significant sequence homology with the yeast sucrose non-fermenting (Snf1) protein (Carling et al, 1994), which is now known to be an orthologue of AMPK (Woods et al, 1994). Snf1, as well as AMPK, regulates ACC function and phosphorylates the SAMS peptide, a specific AMPK substrate. The α1 isoform was later identified and, despite being encoded by a distinct gene, it shares 90% sequence identity within the kinase domain, and 61% elsewhere, compared with α2 (Stapleton et al, 1996). However, the isoforms are not evenly distributed, with α1 being more ubiquitous and present almost equally in all tissues, while α2 is usually less abundant but is preferentially expressed in skeletal muscle, heart and liver. The N-termini of both α subunits contains a kinase domain with a conserved threonine residue (Thr172) in the activation loop, whose phosphorylation by upstream kinases is fundamental for AMPK activation (Hawley et al, 1996). The presence of an autoinhibitory domain (AID) between residues 313-335 of α1 (311-333 of α2) was suggested by an increase in kinase activity when α1 was C-terminally truncated at residue 312 (Crute et al, 1998). A short linker region was identified between the AID

and the C-terminal domain of the α subunit, which appeared to interact with the β and γ subunits (Crute et al, 1998).

1.3.2 The β subunit

The two mammalian AMPK β subunits (β1 and β2), like the α subunits, are encoded by distinct genes and are differentially expressed. β1 levels are higher in the liver and the brain, while β2 is more expressed in skeletal muscle (Thornton et al, 1998). Three gene products in budding yeast, Sip1, Sip2 and Gal83, are closely related to the mammalian β subunits, and they also interact with Snf1, the yeast orthologue of AMPK-α, underlying once more the functional similarity between SNF1 and AMPK complexes (Jiang & Carlson, 1997). AMPK-β subunits contain a carbohydrate-binding module (CBM) in their central part, which causes AMPK to associate with glycogen particles (Hudson et al, 2003; Polekhina et al, 2003). To date the full significance of this interaction is still not clear, although it might be that it helps AMPK to localize close to glycogen-bound substrates, such as glycogen synthase. Another hypothesis is that AMPK itself is regulated by glycogen (Hardie, 2007), as suggested by the inhibitory effect of glycogen on AMPK observed in both purified cell-free systems (McBride et al, 2009) and in skeletal muscle (Wojtaszewski et al, 2002). The C-terminal domain of the β subunits act as a scaffold for assembly of the AMPK complex since it interacts with both the α and the γ subunits (Woods et al, 1996). The AMPK-β subunits are subject to different post-translational modifications. Several phosphorylation sites have been identified, i.e. Ser24/25, Ser108 and Ser182 (Mitchelhill et al, 1997). Ser24/25 and Ser108 are autophosphorylated by the catalytic activity of the α subunits, while the identity of the kinase phosphorylating Ser182 is yet to be determined. It has been reported that phosphorylation of Ser24/25 and Ser182 does not affect AMPK activity

but it is apparently necessary for the nuclear exclusion of β1 (Warden et al, 2001). Phosphorylation of Ser108 does not affect subcellular localization of AMPK but it increases its activity (Sanz et al, 2013). Human AMPK-β subunits contain the consensus motif for myristoylation (M**G**NXXS/T) and both subunits are subject to myristoylation at Gly2 following cleavage of the initial methionine (Martin et al, 2011; Oakhill et al, 2011). In 2008 it was reported that the β subunits of AMPK could also be modified by ubiquitination (Qi et al, 2008) and that this post-translational modification targeted the AMPK complex, or just the β subunit, for proteasomal degradation (Zungu et al, 2011). Finally, it seems that β2 subunit, but not β1, can be sumoylated, and that this results in an increased phosphorylation of Thr172 (Rubio et al, 2013), although the detailed mechanism is yet to be clarified.

1.3.3 The γ subunit

AMPK γ subunits are encoded by three distinct genes and the first isoform to be identified was the one we now know as γ1 (Cheung et al, 2000). The three isoforms are differentially expressed, with γ1 and γ2 being ubiquitously expressed while γ3 seems to be restricted to skeletal muscle. The N-terminal part of the γ subunits is very variable, while the C-terminal regions are highly conserved, with a sequence identity of 63 to 77% (Cheung et al, 2000). These C-terminal regions are characterized by the presence of four tandem repeats of a sequence motif of about 60 amino acids known as cystathione β synthase (CBS) motifs (Bateman, 1997). These repeats associate in pairs to form domains that bind adenosine-containing ligands (Scott et al, 2004). CBS repeats carry a key aspartate residue that is critical for binding of the ribose ring of the bound nucleotide (Kemp et al, 2007). A crystal structure published in 2011 revealed that the four CBS repeats appear to form a flattened disc, where the nucleotides bind

in the clefts between CBS repeats 1 and 2, and CBS repeats 3 and 4 (Xiao et al, 2011). Thus AMPK contains 4 potential nucleotide binding sites which are numbered according to the CBS repeat bearing the conserved aspartate that binds the ribose ring. Site 2 lacks the key aspartate residue and appears to be always empty. Sites 1 and 3 were proposed to reversibly bind AMP or ATP, while site 4 was proposed to contain a permanently-bound AMP molecule (Xiao et al, 2011). In this work it was suggested that site 1 and site 3 have different affinities for AMP or ADP, with site 1 being almost 30-fold stronger than site 3. Xiao and colleagues also identified site 3 as the one responsible for protection against dephosphorylation and thus the one to which ADP also binds. However, it has subsequently been proposed that both site 3 and site 4 account for allosteric stimulation by AMP (Chen et al, 2012). Given the discrepancies mentioned above, it is still not possible to establish a definite model describing the effects of the different binding sites on AMPK activation. Two hereditary conditions, i.e. familial hypertrophic cardiomyopathy (abnormal thickening of the heart walls) and Wolff-Parkinson-White (WPW) syndrome (characterized by ventricular pre-excitation, a premature electrical excitation of the ventricles) are associated with point mutations in the CBS domains of human γ2 subunits (Gollob et al, 2001). These mutations reduce the sensitivity of AMPK to AMP and also the binding of AMP to the CBS domains when introduces into expressed heterotrimers (Daniel & Carling, 2002; Scott et al, 2004). Death during infancy is associated with two more severe mutations: R531Q and R384T (Akman et al, 2007; Burwinkel et al, 2005). All the mutations described show elevated cardiac glycogen content, which may prevent the normal electrical separation of the atria and ventricles, leading to ventricular pre-excitation (Arad et al, 2003; Gollob, 2003; Hardie, 2007). Elevated glycogen storage in skeletal muscle is also observed

when the γ3 subunit presents similar mutations in pigs (Milan et al, 2000) and in humans (Costford et al, 2007).

1.4 AMPK activation by upstream kinases

1.4.1 Phosphorylation by LKB1

Thr172 in the α subunit of AMPK was identified as a fundamental residue for the activation of the kinase, being the target of phosphorylation by upstream kinases. Work in *Saccharomyces cerevisiae* allowed the identification of three kinases (Elm1, Pak1/Sak1 and Tos3) that could activate Snf1 (the yeast homologue of AMPK) in a partially redundant manner, by phosphorylation of Thr210 (equivalent to Thr172 in mammals) (Hong et al, 2003; Sutherland et al, 2003). The catalytic domain of the LKB1 kinase in mammals is closely related to the catalytic domains of these three yeast kinases, suggesting that LKB1 could be the upstream kinase phosphorylating AMPK in mammals. Indeed it was shown that LKB1 could phosphorylate and activate bacterially-expressed AMPK (Hong et al, 2003) and, later it was reported that LKB1 acted in complex with the *pseudokinase* STRAD (Ste-20 related adaptor) and MO25 (mouse protein 25). This heterotrimeric complex was identified to be the major upstream kinase of AMPK (Hawley et al, 2003; Shaw et al, 2004; Woods et al, 2003). LKB1 is a Ser/Thr kinase that is found to be mutated in the Peutz-Jeghers syndrome (PJS) (Alessi et al, 2006). Peutz-Jeghers syndrome is an inherited, autosomal dominant, condition characterized by the formation of benign hamartomatous polyps, especially in the gastrointestinal tract. The binding of LKB1 to STRAD and MO25 is required for its full catalytic activity (Hawley et al, 2003). STRAD and MO25 stabilize the active loop of LKB1 in the conformation needed to phosphorylate its substrates (Zeqiraj et al, 2009a). Being a *pseudokinase*, STRAD does not possess any catalytic activity but still binds ATP,

adopting a closed conformation similar to that of active kinases (Zeqiraj et al, 2009b). Mutations in the ATP-binding pocket of STRAD prevent the full association with MO25 and, consequently, the activation of LKB1 (Zeqiraj et al, 2009b).

LKB1 has been recognized as the master kinase of another 12 AMPK-related protein kinases (ARKs) including NUAK family SNF1-like kinase 1 (NUAK1), sucrose non-fermenting protein-related kinase (SNRK), brain selective kinase1/2 (BRSK1 and BRSK2) or synapses of amphids-deficient kinase (SADK), Salt-inducible kinase1/2/3 (SIK1, SIK2 and SIK3), microtubule affinity regulating kinase1/2/3/4 (MARK1, MARK2, MARK3 and MARK4) or partitioning defective gene 1 (Par1) and maternal embryonic leucine zipper kinase (MELK) (Lizcano et al, 2004). Except for the latter, LKB1 can enhance the kinase activity of ARKs by phosphorylating the threonine residue corresponding to Thr172 in AMPK.

1.4.2 Phosphorylation by CaMKKβ

It has been shown that LKB1 is required to phosphorylate AMPK in response to energy stress (Sakamoto et al, 2005; Shaw et al, 2005). However, it has also been shown that AMPK was still phosphorylated on Thr172 even in LKB1-null cell lines, suggesting the existence of a second kinase phosphorylating AMPK. Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) purified from pig brain had been demonstrated to phosphorylate and activate AMPK in cell-free assays (Hawley et al, 1995), although at that time this was not thought to be the physiologically relevant kinase. The final confirmation that the CaMKK (especially the β isoform) was a second physiologically relevant upstream kinase phosphorylating Thr172, and thus activating AMPK, arrived ten years later (Hawley et al, 2005; Hurley et al, 2005; Woods et al, 2005).

1.5 AMPK regulation by adenosine nucleotides: promotion of phosphorylation

Moore et al (1991) showed that when rat hepatocytes were treated with 20 mM fructose, reciprocal changes in the activities of AMPK and ACC were observed over time. When they measured the AMP:ATP ratio following the addition of fructose, they observed a transient increase that then returned to basal values with time, accompanied by a transient activation of AMPK and a transient inactivation of ACC. While the inactivation of ACC could be explained by allosteric stimulation of AMPK by AMP, the increase in AMPK activity could not be due only to allosteric stimulation, because such changes would not have been observed in a cell lysate. Therefore, changes in AMPK phosphorylation must have occurred. This was the first evidence that the phosphorylation of AMPK could be mediated by nucleotide levels. Further confirmation arrived in 1994 when Weekes et al (1994) separated AMPK activity from the activity of its upstream kinase and showed that phosphorylation of AMPK was dependent upon the distinct upstream kinase and that this was stimulated by AMP. An important breakthrough came when Thr172 was recognized as the target site for the upstream kinase on the AMPK α subunit (Hawley et al, 1996). However, since 1991 the role of AMP to promote Thr172 phosphorylation has been discussed and challenged. In 2010 it was proposed that promotion of phosphorylation by AMP could be achieved only when the β subunit was myristoylated on Gly2 (Oakhill et al, 2010). Mutation of Gly2 did not seem to affect either allosteric stimulation of AMPK or the ability of AMP to protect against dephosphorylation. However, Thr172 phosphorylation, induced by glucose deprivation, and the consequent membrane association of wild-type AMPK were lost with the non-myristoylatable G2A mutant (Oakhill et al, 2010). The myristoyl

group in the β subunit promote association of AMPK with cellular membranes (Warden et al, 2001) and it is known that a number of AMPK substrates associate with the membrane. A model for AMPK regulation, dependent on its β subunit myristoylation was proposed. High levels of ATP were proposed to induce the association between the myristoyl group and the kinase domain, preventing the access of upstream kinases to Thr172. When cells experience stress conditions, AMP was proposed to displace ATP at the γ subunit, causing the release of the myristoyl group and promoting AMPK membrane association (thus targeting some of its substrates) and Thr172 phosphorylation by the upstream kinases (Oakhill et al, 2010). When ATP returns to basal levels, the mechanism is reversed.

1.6 AMPK regulation by adenosine nucleotides: allosteric stimulation and protection from dephosphorylation

The level of AMP in the cell seems to be determined by the activity of the enzyme adenylate kinase (Dzeja & Terzic, 2003) which catalyses the reversible interconversion of the three adenine nucleotides: $2ADP \leftrightarrow ATP+AMP$. Adenylate kinase appears to have a high basal activity in almost all eukaryotic cells, so that the mass action ratio ($[ATP]\bullet[AMP]/[ADP]^2$) is always close to the equilibrium value of around 1. If this reaction is at equilibrium, the cellular AMP/ATP ratio will vary as the square of the ADP/ATP ratio so that the increase in AMP, occurring whenever ATP/ADP falls, will be much larger than the changes in ATP or ADP (Hardie & Hawley, 2001). So, when the cells experience metabolic stress that causes the ATP/ADP ratio to fall, cellular AMP concentration can be used as sensitive indicator of such energy stress. Binding of AMP to AMPK γ-subunits causes >10 fold allosteric stimulation (the regulation of a protein by binding of an effector molecule at a site other than the active site, usually caused by

a conformational change). It was also demonstrated that binding of AMP to AMPK protected the kinase from dephosphorylation of Thr172 by protein phosphatases (Davies et al, 1995). When this effect was analysed, it was found that the IC_{50} for the effect of AMP on dephosphorylation was very similar to the EC_{50} for the allosteric stimulation. Also, the effect was mimicked by AMP analogues that allosterically stimulated AMPK activity and was not specific for a particular phosphatase. Finally the activity of the phosphatases themselves was not affected by AMP. All these lines of evidence suggested that the effect of AMP on inhibition of dephosphorylation is substrate-mediated. The protection from dephosphorylation mediated by AMP was further confirmed when it was shown that mutation of the nucleotide binding sites of the γ subunits abolished it (Sanders et al, 2007b).

1.7 AMPK regulation by ADP

In 2011 Xiao and colleagues suggested that the protection against dephosphorylation mediated by the binding of AMP to AMPK could be mimicked by the binding of ADP (Xiao et al, 2011). It was also proposed that ADP could promote phosphorylation of Thr172 by both LKB1 and CaMKKβ (Oakhill et al, 2010; Oakhill et al, 2011). These findings, together with the fact that ADP concentration in the cell is usually up to one order of magnitude higher than that of AMP, raised the possibility that ADP, not AMP was the physiological signal promoting AMPK activation. This question was investigated by Gowans and co-workers (Gowans et al, 2013). In this study it was demonstrated that dephosphorylation of Thr172 by the phosphatase PP2Cα was prevented by both ADP and AMP. However AMP was 10-fold more potent than ADP. It was also confirmed that phosphorylation of Thr172 by LKB1 was promoted by AMP, but not ADP, while phosphorylation by CaMKKβ was not dependent on either AMP or

ADP. According to their results, while ADP appeared to have a role in AMPK regulation, AMP was still likely to be the critical physiological activator of AMPK, so that the name AMP-activated protein kinase remained appropriate.

A summary of the mechanisms that regulate AMPK activation is presented in Figure 1.2.

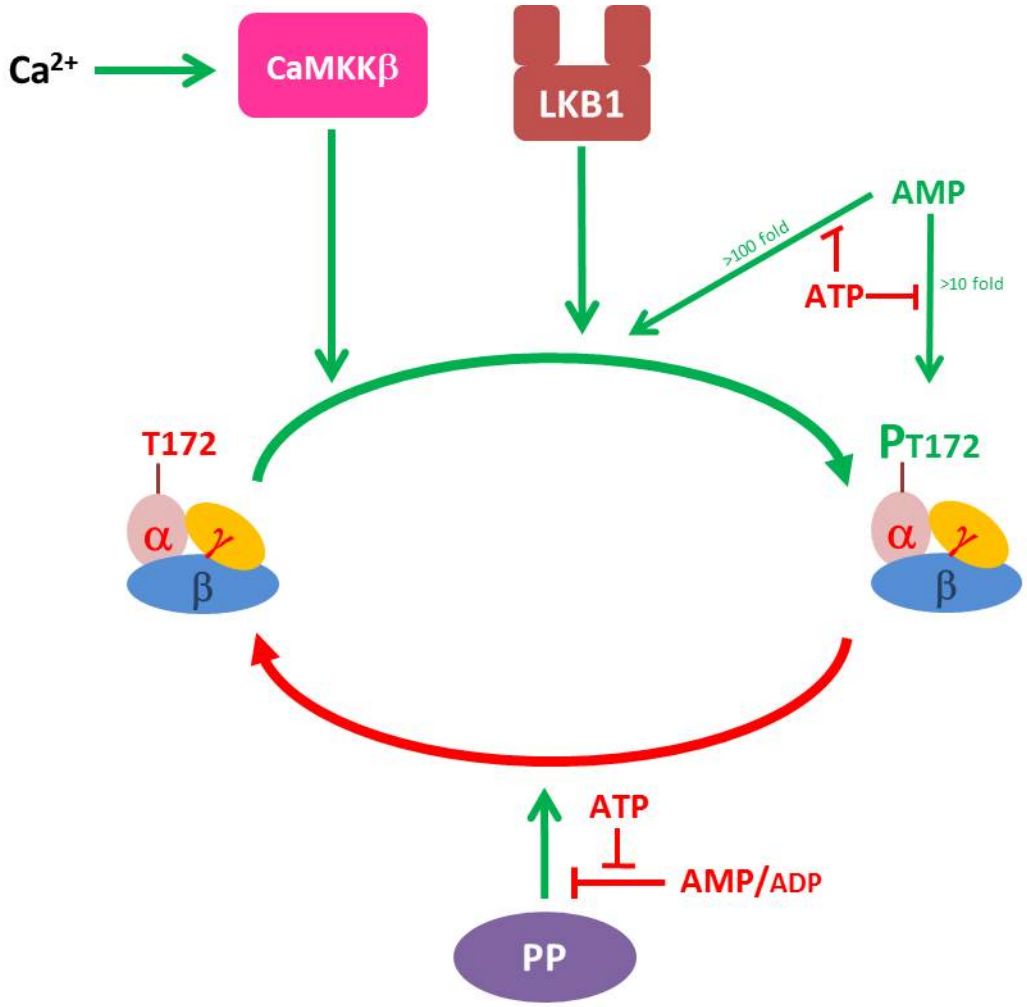

Figure 1.2. Regulation of AMPK. Schematization of the mechanisms by which AMPK is regulated by AMP, ADP and ATP. 1) Promotion of phosphorylation by upstream kinases; 2) Protection from dephosphorylation; 3) Allosteric stimulation.

1.8 AMPK activators

As a sensor of cellular energy stress, AMPK is mostly activated by those processes that interfere with the levels of ATP in the cell. Processes that increase ATP consumption (i.e. muscle contraction during exercise) or that decrease ATP production (i.e. ischaemia or hypoxia) lead to an increase in both AMP:ATP and ADP:ATP ratios, thus activating AMPK. A variety of drugs and xenobiotics (Hardie et al, 2012a), as well as a number of hormones (Kahn et al, 2005), are also known to promote AMPK activation. In the following sections the different mechanisms by which AMPK is activated are described (Fig.1.3)

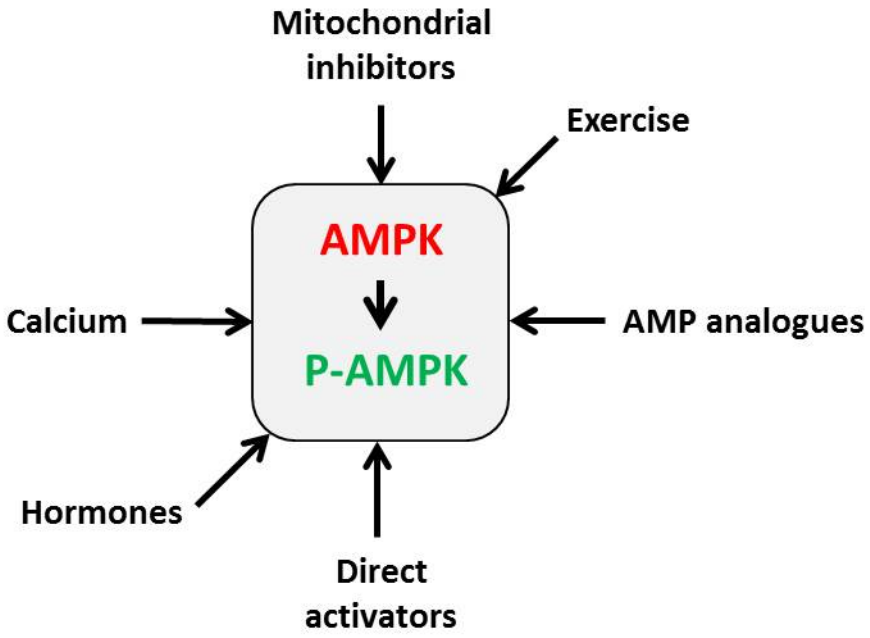

Figure 1.3. Activators of AMPK. AMPK is activated by a large number of different drugs and xenobiotics as well as by hormones and exercise.

1.8.1 Exercise activates AMPK

When skeletal muscles contract during exercise, the intracellular glucose transporter GLUT4 is translocated to the cell surface, and the level of glucose uptake increases

(Merry & McConell, 2009). Muscle contraction, causing ATP depletion, activates AMPK, which is then responsible for the increase of glucose uptake (as explained in more details in section 1.9.1) (Hayashi et al, 1998). It has been suggested that AMPK activation by exercise is mediated by LKB1. In mice lacking LKB1 in skeletal muscle, AMPK-α2 activity was dramatically reduced and could not be increased by muscle contraction. In the same way, also glucose uptake was inhibited and phosphorylation of Acetyl-CoA carboxylase 2 (ACC2), a downstream target of AMPK, was greatly reduced, underlying the central role of LKB1 in AMPK activation by exercise (Sakamoto et al, 2005) . It has also been shown that AMPK is the only ARK downstream of LKB1 responding to muscle contraction, further confirming that the effects on glucose uptake in skeletal muscle are mediated by AMPK rather than another ARK (Sakamoto et al, 2004). It has been proposed that AMPK was also responsible for many of the beneficial effects of exercise, especially increased mitochondrial biogenesis (Zong et al, 2002) and increased fatty acid oxidation (Aschenbach et al, 2004)

1.8.2 Activation of AMPK by hormones

Adipocytes secrete a range of hormones collectively termed adipokines and it has been shown that AMPK is regulated by more than one of them (Kahn et al, 2005). The first example is the hormone ghrelin, that is released from the gut during fasting or starvation and, once in the hypothalamus, it acts as a “hunger signal” to increase appetite. Ghrelin receptors are located in the arcuate nucleus of the hypothalamus together with NPY/AgRP neurons, that are required for ghrelin to promote feeding (Luquet et al, 2007). It has been suggested that ghrelin may not act on NPY/AgRP neurons themselves, but on presynaptic neurons upstream. Activity of these neurons was indirectly assessed by measuring the frequency of postsynaptic currents in the

NYP/AgRP neurons (Yang et al, 2011). Pharmacological analysis of these currents suggests that ghrelin activates the G-coupled receptor GHSR1, stimulating the production of IP_3 and the consequent release of Ca^{2+} in the presynaptic neurons. Ca^{2+} then triggers AMPK activation via CaMKKβ. As a confirmation of this hypothesis, increases in food intake induced by administration of ghrelin, were absent in mice with whole-body knock-out of CaMKKβ (Anderson et al, 2008).

The hormone leptin, known also as "satiety hormone", is produced in proportion to the fat levels of the body and released in large amounts in obese individuals. Leptin was shown to rapidly but transiently increase activation of AMPK, enhancing fatty acid oxidation in skeletal muscle (Minokoshi et al, 2002). However, in the hypothalamus, leptin has been reported to inhibit AMPK-α2 (Minokoshi et al, 2004). It has been suggested that leptin triggers the release of an opioid (possibly β-endorphin) from the proopiomelanocortin (POMC) neurons, which then inhibits AMPK via μ-opioid receptors in the presynaptic neurons upstream of NPY/AgRP neurons. By interrupting the positive feedback loop set up by ghrelin, leptin can terminate the stimulation of NYP/AgRP neurons and thus feeding (Yang et al, 2011). It is then possible to picture the possible scenario in which leptin and ghrelin, along with insulin, play critical roles in order to regulate food intake and energy expenditure via neuronal circuits. During fasting or starvation ghrelin increases while insulin, usually released from pancreatic β-cells in response to hyperglycaemia, and leptin decrease. This will result in increased AMPK activity in the presynaptic neurons, increased activity of NPY/AgRP neurons and decreased activity of POMC neurons, driving an orexigenic response. On the other hand, during a positive energy balance, leptin and insulin levels will rise while ghrelin levels will decrease, leading to AMPK inhibition and, thus, increasing anorexigenic

outputs. This mechanism seems to fail in obese individuals, who apparently become resistant to both leptin and insulin in the hypothalamus. In this condition ghrelin can escape the restraining influence of leptin and insulin, chronically activating AMPK in the hypothalamus and thus inappropriately activating NPY/AGRP neurons. This would maintain a prolonged hunger signal despite a positive energy balance (Hardie & Ashford, 2014).

The hormone adiponectin is released from adipocytes in larger amounts in lean individuals, suggesting that it is released when fat stores are low. Binding of adiponectin to its receptor AdipoRI activates AMPK (even if the precise mechanism of activation is not yet clear) and promotes fat oxidation in liver and muscle, while inhibings glucose production in the liver (Kadowaki & Yamauchi, 2005). In the hypothalamus, adiponectin activates AMPK, mimicking the effect of ghrelin but opposing the effect of leptin (Kubota et al, 2007).

The thyroid hormone tri-iodothytonine (T3) has an effect in almost all cells. However, its effect on whole-body energy balance appears to involve inhibition of AMPK in neurons of the ventromedial hypothalamus (Lopez et al, 2010). This inhibition triggers the release of norepinephrine and/or epinephrine in the periphery and increase energy expenditure by stimulating the release of fatty acids from adipose tissue and by promoting fat oxidation and heat production in brown adipose tissue (Lopez et al, 2010).

1.8.3 Activation of AMPK by drugs and xenobiotics

A vast range of compounds had been shown to activate AMPK, using a variety of mechanisms. In 2010 a study was conducted to generate a straight forward technique that would easily allow identification of the mechanism by which each compound

activated AMPK (Hawley et al, 2010). In this study HEK-293 cells were modified in order to stably express a mutated form of the γ2 subunit of AMPK. Mutation of Arg531 in AMPK-γ2 (located in CBS repeat 4) to Gly (RG mutant), found in some cases of WPW syndrome, prevents nucleotide binding at site 3 and renders AMPK insensitive to changes in cellular nucleotide levels. Thus, when a compound only activates the wild-type γ2-containing complexes, it means that its mechanism of activation relies on changes in cellular nucleotides. This class of compounds was demonstrated to disrupt energy status by increasing both the ADP:ATP and AMP:ATP ratios, in most cases by altering mitochondrial function (Hawley et al, 2010). Other compounds are instead able to activate AMPK also when the RG mutation is present in the γ2 subunit and their mechanism of activation is clearly AMP-independent (Hawley et al, 2010).

1.8.3.1 Mitochondrial inhibitors

Many of the compounds found to activate AMPK are metabolic poisons that either affect glycolysis or, more often, mitochondrial activity. In doing so, they inhibit ATP synthesis and activate AMPK indirectly by increasing cellular AMP levels. Several different compounds belong to this class of activators. The first one worth to be mentioned is metformin. Metformin is a widely prescribed drug, used as a first-choice treatment for type 2 diabetes (Rena et al, 2013). Along with the related compound phenformin, it derives from galegine, a natural product of the plant *Galega officinalis* whose extracts had been used for centuries to treat symptoms of diabetes (Hardie et al, 2012a). Both galegine and phenformin had been shown to be also very potent activators of AMPK (Hawley et al, 2003; Mooney et al, 2008). Metformin was shown to activate AMPK in intact cells, leading to decreased hepatic glucose production and increased glucose uptake in muscle (Zhou et al, 2001). However it was clear that this

activation was indirect since no effect of metformin could be observed on *in vitro* AMPK activity (Hawley et al, 2002). It had been shown that metformin inhibits complex I of the mitochondrial chain (El-Mir et al, 2000) suggesting that the mechanism by which it activates AMPK could be dependent on changes in cellular nucleotides. This hypothesis was confirmed in the study conducted by Hawley and colleagues (Hawley et al, 2010) where it was demonstrated that metformin failed to activate AMPK containing the RG-γ2 mutant. In the same study, phenformin and galegine were shown to activate AMPK through the same mechanism. However some discrepancies have emerged about the role of AMPK as a mediator of the beneficial effects of metformin. The effect of metformin was reversed when compound C, an AMPK inhibitor was used (Zhou et al, 2001). However, compound C appeared to be a non-specific inhibitor of AMPK, having different off-target effects (Bain et al, 2007). Also, mice with both catalytic subunits of AMPK deleted from the liver displayed normal acute effects of metformin on hepatic glucose production, as did hepatocytes from mice lacking either AMPK or LKB1 (Foretz et al, 2010). Metformin was also claimed to inhibit mTORC1 signalling and to stimulate glucose uptake in an AMPK-independent manner (Kalender et al, 2010; Turban et al, 2012). However, a recent study has suggested that AMPK actually has a crucial role in the response to metformin. Mice carrying alanine knock-in mutations at the sites phosphorylated by AMPK in both ACC1 (Ser79) and ACC2 (Ser212) displayed increased hepatic lipogenesis and decreased fatty acid oxidation compared to wild-type mice. This resulted in elevated hepatic lipid content which was associated with insulin resistance. Indeed these mice were found to be hyperglycaemic, hyperinsulinemic, glucose intolerant and insulin resistant. However, they were not obese (Fullerton et al, 2013). If mice underwent a high-fat diet and became obese, long-term treatment with metformin reduced hepatic lipid content and

improved insulin-sensitivity in wild-type mice but not in the double knock-in mice. It therefore seems that phosphorylation and inhibition of ACC by AMPK is fundamental in mediating the long-term beneficial effects of metformin (Fullerton et al, 2013). Also, it is worth noting that pre-treatment of hepatocytes with low concentrations of metformin (80 μM was the highest concentration used, and is the highest concentration found in the portal vein) resulted in the suppression of glucose production and gluconeogenic gene expression. This effect occurred without any increase in AMP/ATP ratio but, at the same time, was mediated by an increase in AMPK phosphorylation, possibly suggesting and additional direct effect of metformin on AMPK activation (Cao, J. et al. 2014).

Troglitazone, pioglitazone and rosiglitazone belong to the class of thiazolidinediones (TZDs), which have been used as antidiabetic drugs, and were shown to increase AMPK activity (Fryer et al, 2002; LeBrasseur et al, 2006; Saha et al, 2004). TZDs are also known to activate AMPK through indirect mechanisms. Firstly, they bind to the transcription factor PPAR-γ, causing its activation. As a consequence of this, adiponectin, an hormone known to activate AMPK, is released (Yamauchi et al, 2002). Most of the beneficial effects of low doses of TZDs on diabetes were abolished by knocking out adiponectin, confirming this mechanism of action (Kubota et al, 2006). However, at higher concentrations, TZDs have also been shown to inhibit mitochondrial function (Fryer et al, 2002; Hawley et al, 2010), causing rapid glucose uptake and fatty acid oxidation that could be AMPK-dependent and are unlikely to be explained by changes in transcription (LeBrasseur et al, 2006).

In nature, there are numerous plant products that have been demonstrated to promote AMPK activity, many of which have been used in traditional medicines. Some

examples are resveratrol, present in red wine and grapes (Baur et al, 2006), berberine, from Chinese Goldthread (Turner et al, 2008) and quercetin, found in fruits and vegetables (Ahn et al, 2008). The precise mechanism of activation of all these different compounds remains uncertain, although it has been proposed that some of them also activate AMPK by impairing mitochondrial function (Turner et al, 2008). Indeed resveratrol, berberine and quercitin all failed to activate the AMPK containing the RG-γ2 mutant, confirming that their mechanism of activation is indirect and dependent on changes in cellular nucleotides (Hawley et al, 2010).

1.8.3.2 AMP analogues

Another class of AMPK activators are pro-drugs that are converted following cellular uptake into AMP analogues, which activate AMPK by mimicking the effect of AMP binding (Hardie, 2015). The nucleoside 5-aminoimidazole-4-carboxamide riboside (AICAR) belongs to this class of compounds, and was also the first compound identified to activate AMPK in intact cells and *in vivo*. Once in the cell, AICAR is converted to the monophosphorylated form 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP) (Sabina et al, 1985). ZMP activates AMPK by allosteric stimulation and by protection against dephosphorylation (Corton et al, 1995). Since ZMP binds to the same site as AMP, treatment of cells with AICAR does not have any effect on AMPK containing the RG-γ2 mutant (Hawley et al, 2010) and it has been extensively used as a tool to characterize the downstream effects of AMPK. However, being an AMP mimetic, ZMP is also able to bind to other AMP-sensitive enzymes such as fructose-1,6-bisphosphatase, to reduce gluconeogenesis (Vincent et al, 1991), and to glycogen phosphorylase, to increase glycogenolysis (Longnus et al, 2003). Despite the fact that AICAR has been shown to reverse some of the defects associated with the

metabolic syndrome (Song et al, 2002), this lack of specificity, very low oral availability and rapid metabolism renders it unsuitable for clinical use.

For many years, AICAR was the only member of this class of compounds. Recently a new AMP analogue, 5-(5-hydroxyl-isoxazol-3-yl)-furan-2-phosphonic acid or C2, was reported to activate AMPK and to be a 1000-fold more potent activator than ZMP in cell-free assays (Gomez-Galeno et al, 2010). It was identified as a potent activator after screening a library of 1200 AMP mimetics against both human and rat AMPK, by monitoring the phosphorylation of SAMS peptide (Davies et al, 1989). Unlike AICAR, C2 does not affect other AMP-sensitive metabolic enzymes and most kinases, including members of the AMPK-related kinase family and AMPK upstream kinases, were not significantly affected by low doses of C2 either (Hunter et al, 2014). Cell-free kinase assays showed that C2 activates AMPK both allosterically and by protecting Thr172 from dephosphorylation. Also, it has a preference for α1-containing complexes (Hunter et al, 2014). C2 carries a negatively charged phosphonate group and is not cell permeable. However, it can be administered to cells as a pro-drug called C13, in which the phosphonate group is esterified, with C13 being converted into C2 by intracellular esterases. C13 has been shown to activate AMPK in a concentration-dependent manner in isolated mouse hepatocytes, leading to the inhibition of both *de novo* lipid synthesis and fatty acid esterification. Both effects were lost in AMPK-KO hepatocytes (Hunter et al, 2014). Although the precise binding site of C2 has not been identified, it is a structural analogue of AMP. Treatment of cells with C13 did not affect the levels of cellular nucleotides, and no increase in AMPK activity could be observed when RG-γ2 expressing cells where treated with the compound (Hunter et al, 2014). These lines of evidence strongly suggest that C2 binds to the same site(s) as AMP, and it can be

considered the second member of the class of AMPK activators that are AMP analogues.

1.8.3.3 Direct activators

A third class of activators is comprised of molecules that bind directly to AMPK. There are currently four compounds belonging to this group: A769662, salicylate, 991 and MT63-78. The first direct activator identified was the thienopyridone A769662 (Cool et al, 2006). Like AMP, A769662 has been found to allosterically activate AMPK and to protect Thr172 from dephosphorylation (Goransson et al, 2007; Sanders et al, 2007a). Despite sharing some of the same effects, A769662 and AMP do not bind at the same site to promote AMPK activation. The A769662 binding site involves the β subunit of AMPK and particularly its residue Ser108. Thus, an S108A mutation that prevents autophosphorylation of that serine residue, almost completely abolished AMPK activation by A769662 (Scott et al, 2008). Furthermore, the compound has a preference towards β1-containing complexes, since it almost completely fails to activate AMPK-β2 complexes (Hawley et al, 2012; Scott et al, 2008). A769662 is one of the few compounds that also activates AMPK containing the RG-γ2 mutant, confirming its AMP-independent mechanism of activation (Hawley et al, 2010). Although it has the ability to directly activate AMPK, A769662 has poor oral solubility (Cool et al, 2006) and may possess AMPK-independent functions (Moreno et al, 2008) which may limit its use as a therapeutic agent.

A second compound that has been found to directly activate AMPK is salicylate. Among the four direct activators, salicylate is the only natural compound. It is present in willow bark but it is also produced by other plants as a defence mechanism in response to infection. For millennia it has been used in traditional medicine. Nowadays its

medical usage has been largely replaced by aspirin or by salsalate, which are both broken down to form salicylate after oral administration. Aspirin is known to inhibit prostaglandin synthesis by inhibiting the cyclo-oxygenases COX1 and COX2 (Vane, 1971). However salicylate has recently been shown to directly activate AMPK in a manner independent of changes in cellular energy state (Hawley et al, 2012). Like A769662, salicylate also requires phosphorylation of Ser108 to effectively activate AMPK, and activates β2-containing complexes very poorly. Salicylate can modestly stimulate AMPK activity allosterically, although its major effect is to protect Thr172 from dephosphorylation by protein phosphatases. It was shown that salicylate can antagonize allosteric stimulation by A769662, suggesting that they compete for binding at the same site. Salicylate treatment *in vivo* can cause a switch from carbohydrate to fat utilization and can decrease the serum levels of fatty acids. Both of these effects require the presence of the β1 isoform of AMPK, confirming that these metabolic effects are mediated by AMPK activation (Hawley et al, 2012).

Recently two other compounds have been identified to join the group of direct activators. The small molecule MT63-78 was identified in a targeted screen using purified human recombinant AMPK and, using cell free assays, it was proven to allosterically activate AMPK in a dose-dependent manner (Zadra et al, 2014). Like AMP, A769662 and salicylate, MT63-78 also protects AMPK from dephosphorylation by protein phosphatases and shows a preference for β1-containing complexes. However, *in vitro* experiments show that high doses of MT63-78 were also able to activate β2-containing AMPK complexes (Zadra et al, 2014).

The fourth and possibly the most potent of the AMPK direct activators is a cyclic benzimidazole derivative developed by Merck Sharp and Dohme Corporation and

Metabasis Therapeutics, called 991. This compound was shown to be 5-10 fold more potent than A769662 in assays monitoring allosteric stimulation and protection against dephosphorylation (Xiao et al, 2013). Cell-based assays to determine the effectiveness of 991 showed activation at much lower concentrations than required for A769662. Like the other direct activators, 991 binds β1-containing AMPK 10 times more tightly than β2 complexes, without losing the ability to activate the latter. Recently, it has been showed that 991 binds in the same site as A769662 (Xiao et al, 2013). Additional studies showed that 991 (also known as ex229) dose-dependently increased AMPK activity in rat epitrochlearis muscle and, at a concentration of 100 μM, AMPK activation was comparable to that observed after muscle contraction. It also caused a 2-fold increase in glucose uptake, which, along with ACC phosphorylation, was abolished in AMPK α1/α2 double knock-out myotubes incubated with 991 (Lai et al, 2014).

The presence of a well-defined binding site for all of these direct activators raises the intriguing questions as to where this binding site is located and whether there are natural ligands that bind this site. These questions will be further investigated in the following chapters of this thesis.

1.8.3.4 Calcium

It has been demonstrated that AMPK can be phosphorylated at Thr172 by CaMKKβ, a kinase that is activated in response to rising levels of calcium (Hawley et al, 2005). Hawley and colleagues demonstrated that silencing CaMKKβ using targeting siRNAs greatly reduced AMPK activation by the calcium ionophore A23187, confirming that the expression levels of CaMKKβ were critical for AMPK activation by calcium to occur. The same outcome was obtained when CaMKKβ was inhibited with the inhibitor

STO609. Pharmacological agents that increase the level of calcium in the cell can therefore significantly activate AMPK. Examples in this regards are the calcium ionophore A23187 (Hawley et al, 2010), ionomycin (Hurley et al, 2005) and also, in muscle, caffeine (Jensen et al, 2007).

1.9 Downstream events regulated by AMPK

Once activated, AMPK acts as a sensor of cellular energy status, in order to re-establish energetic balance when it is disrupted during cellular stress conditions. To accomplish this, AMPK promotes catabolic pathways that generate ATP and, at the same time, switches off anabolic pathways that consume ATP. It achieves this by directly phosphorylating metabolic enzymes or by regulating transcription factors or co-activators (Hardie, 2015). Some downstream effects of AMPK are summarised in Figure 1.4. AMPK phosphorylates downstream targets at Ser/Thr residues located in a sequence motif characterized by hydrophobic residues at the -5 and +4 positions and basic residues at -4 or -3, or both (Hardie et al, 2012b). An additional basic residue at -6 increases the specificity and the best substrates for AMPK (such as ACC1 and ACC2) also have additional hydrophobic residues forming an amphipathic helix N-terminal to the -5 position.

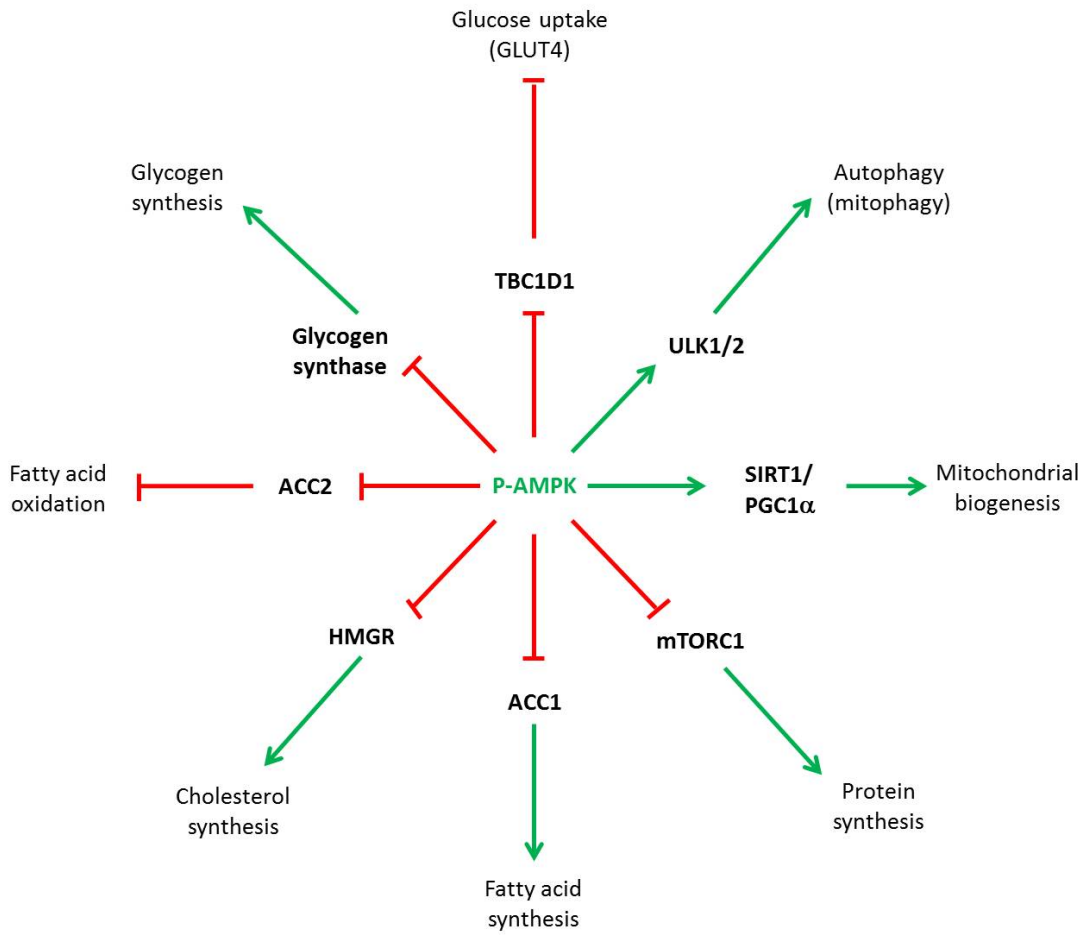

Figure 1.4. Some downstream targets and effects of AMPK. AMPK senses changes in cellular energetic status and, in order to maintain it, promotes catabolic pathways producing ATP while inhibiting anabolic pathways consuming ATP.

1.9.1 Glucose metabolism

During muscle contraction, blood glucose is used to generate ATP, and activated AMPK appears to mediate the increase in glucose uptake that occurs during this process. Enhanced glucose uptake is achieved by translocation of the glucose transporter GLUT4 from intracellular storage vesicles to the plasma membrane. These vesicles move to the plasma membrane in a process mediated by members of the Rab family of G-proteins in their GTP-bound state (Sakamoto & Holman, 2008). Under basal conditions, Rabs are kept in the inactive GDP-bound state by TBC1D4 and/or TBC1D1,

which contain Rab-GTPase activator protein (Rab-GAP) domains and are associated with vesicles containing GLUT4. The insulin-activated kinase Akt phosphorylates TBC1D4, triggering association with 14-3-3 proteins and consequent dissociation from the storage vesicles (Geraghty et al, 2007). In the same way, AMPK phosphorylates TBC1D1, leading to a similar outcome (Chen et al, 2008). In cell-free assays it seems that AMPK can phosphorylate both Ser237 and Thr596 of TBC1D1. However in intact cells, only Ser237 appears to be phosphorylated in response to AMPK activation, suggesting that other kinases may be involved in Thr596 phosphorylation and hence GLUT4 translocation (Chen et al, 2008). Dissociation of both TBC1D1 and TBC1D4 promotes the conversion of Rabs to their GTP-bound form, with the consequent transport and subsequent fusion of GLUT4with the plasma membrane. It is still not clear whether AMPK entirely mediates the effect of muscle contraction on glucose uptake. For example, in mice with a knock-out of the AMPK-α2 subunit, AICAR had no effect on glucose uptake but the effects of muscle contraction were normal. When the AMPK-α1 subunit was deleted there were no changes in the response to either AICAR or muscle contraction (Jorgensen et al, 2004b). However, experiments performed by genetically depletion of only one AMPK isoform still leaves open the possibility that there might be residual AMPK activity, sufficient to increase glucose uptake during muscle contraction. Mice lacking both β1 and β2 subunits showed reduced muscle glucose uptake during treadmill exercise and muscle contraction (O'Neil et al., 2011). Recently also mice lacking both α1 and α2 subunits of AMPK have been characterized and they were found to have a reduction in contraction-stimulated glucose uptake in the soleus but not extensor digitorium longus muscle (Lantier et al., 2014). On the other hand, when mice lacked LKB1 in muscle, glucose uptake was not enhanced significantly either by AICAR or by contraction (Sakamoto et al, 2005).

AMPK can also promote glucose uptake in cells other than those in muscle which express a different glucose transporter, GLUT1. GLUT1 is already located in the plasma membrane and it seems that AMPK promotes glucose uptake by activating GLUT1 (Barnes et al, 2002). Barnes and colleagues showed that in the rat liver epithelial cell line Clone 9 metabolic and osmotic stress leaded to increased glucose uptake. This increase was not paralleled by changes in cell-surface levels of GLUT1. However, they showed that the increased glucose transport seen in Clone 9 cells correlated with increased exofacial labelling of GLUT1 and was also enhanced by treatment with AICAR. Recently a mechanism for this activation was suggested. Binding of GLUT1 to TXNIP, an α-arrestin family protein that is induced by glucose elevation, promotes GLUT1 internalization through clathrin coated pits. AMPK has been found to phosphorylate TXNIP, enhancing its rapid degradation and thus preventing the internalization of GLUT1. This eventually leads to increased glucose uptake (Wu et al, 2013).

The main storage form of glucose in the body is glycogen. When the energy balance of the cell is affected, glucose needs to be used to produce ATP. To this aim, apart from enhancing glucose uptake, AMPK also phosphorylates and inhibits the enzyme producing glycogen, glycogen synthase, thus decreasing the rate of glycogen synthesis (Jorgensen et al, 2004a) and preventing storage of “precious” glucose. Mammalian glycogen synthase exists as two isoforms, muscle glycogen synthase (GYS1) and liver glycogen synthase (GYS2). The first is widely expressed while the liver isoform expression is entirely restricted to the liver. Glycogen synthase(s) catalyses the transfer of the glucose moiety of UDP-glucose to the hydroxyl at C4 of the terminal residue of a glycogen chain, thus promoting the elongation of the chain itself. Glycogen synthase(s)

contain clusters of phosphorylation sites on both the N and C termini. The largest effects on glycogen synthase activity were observed by phosphorylation of site 2, in the N-terminal region, and site 3a, in the C-terminal region. While site 3a, as well as 3b, 3c and 4 are phosphorylated by glycogen synthase kinase-3 (GSK3), AMPK appears to be the major kinase acting on site 2 in GYS1 (Carling & Hardie, 1989; Jorgensen et al, 2004a). Site 2 was identified as Ser7 (in both isoforms), a residue that lies in a favourable consensus motif for phosphorylation by AMPK (Bultot et al, 2012). When AMPK phosphorylates Ser7, glycogen synthase affinity for UDP-glucose and glucose-6-phosphate decreases dramatically, leading to the enzyme inactivation. When isolated rat hepatocytes were incubated with AICAR, phosphorylation of Ser7 could be observed along with a persistent inactivation of glycogen synthase. By contrast, inactivation of glycogen synthase by glucagon was transient. In mice carrying a specific deletion of $\alpha 1/\alpha 2$ subunits in the liver, inactivation of glycogen synthase by AICAR was blunted while glucagon continued to inactivate the enzyme. These results provided evidence that phosphorylation of Ser7, mediated by AMPK, inactivates glycogen synthase (Bultot et al, 2012)

AMPK can down-regulate the expression of some gluconeogenic genes, including glucose-6-phosphatase, by regulating the activity of transcriptional co-activators, such as CRTC2 (CREB-regulated transcription co-activator 2) (Koo et al, 2005). Down-regulation of the expression of gluconeogenic genes seems to be achieved also indirectly by AMPK. FOXO transcription factors promote the expression of gluconeogenic genes and they are activated by class IIa histone deacetylases. It has been reported that AMPK can inhibit the function of these deacetylases by promoting their nuclear exclusion (Mihaylova et al, 2011).

AMPK has been shown to also increase the rate of glycolysis through the phosphorylation of isoforms of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB), the enzyme catalysing the generation and breakdown of fructose-2,6-bisphosphate, a potent activator of the glycolytic enzyme 6-phosphofructo-1-kinase. However, this form or regulation has been observed only in cells expressing the PFKFB2 isoform, such as cardiac myocytes (Marsin et al, 2000), or the PFKFB3 isoform, such as monocytes, macrophages and certain cancer cells (Marsin et al, 2002).

1.9.2 Lipid metabolism

The role of AMPK in lipid metabolism is mainly to reduce fatty acid and cholesterol synthesis while promoting fatty acid oxidation. ACC is the enzyme that catalyses the conversion of acetyl-CoA into malonyl-CoA, one of the first steps of fatty acid synthesis. There two isoforms of ACC, ACC1 and ACC2, and both of them are phosphorylated and inhibited by AMPK. Phosphorylation on Ser79 of ACC1 reduces its catalytic activity, therefore inhibiting fatty acid synthesis (Davies et al, 1992; Davies et al, 1990). ACC2 is associated with mitochondrial membranes and is thought to increase malonyl-CoA levels at the mitochondrial surface (Abu-Elheiga et al, 2000). Malonyl-CoA inhibits carnitine:palmitoyl-CoA transferase 1 (CPT1), the enzyme mediating the entry of long-chain fatty acids into mitochondria for fatty acid oxidation. By inhibiting ACC2, AMPK reduces levels of malonyl-CoA and consequently promotes fatty acid oxidation (Merrill et al, 1997). However, genetic disruption of ACC1 and ACC2 showed confliction results about the roles of these enzymes in controlling fatty acid metabolism. Specific knock out of ACC1 in the livers was compensated by ACC2 activity, and the *de novo* lipogenesis rate was not significantly altered (Harada, N. et al, 2007). Also, knock out of

ACC2 had no effect on total malonyl-CoA content and fatty acid oxidation rates in skeletal muscle, suggesting a metabolic compensation in response to the loss of ACC2 (Olson, D.P. et al, 2010). Mice with alanine knock-in mutations in ACC1 (at Ser79) and ACC2 (at Ser212) showed elevated lipogenesis and lower fatty acid oxidation, without a clear separation of the individual roles of ACC1 and ACC2, suggesting that their activities might be redundant (Fullerton et al, 2013). AMPK also downregulates expression of ACC1 and other lipogenic enzymes, probably through the phosphorylation of the lipogenic transcription factor sterol regulatory element-binding protein 1C (SREBP1C) (Li et al, 2011).

The enzyme HMG-CoA reductase, crucial for cholesterol synthesis, is also phosphorylated and inactivated by AMPK (Clarke & Hardie, 1990), while its transcription, mediated by SREBP2, is also down-regulated by AMPK (Liu et al, 2015).

1.9.3 Protein metabolism

AMPK plays a role in regulating protein synthesis and cellular growth, and it does so by inhibiting the mammalian target of rapamycin (mTOR) pathway (Gwinn et al, 2008). The mTOR complex 1 (mTORC1) controls protein synthesis and is composed of mTOR, the regulatory-associated protein of mTOR (Raptor) and other subunits. mTORC1 phosphorylates the eukaryotic translation initiation factor 4E-binding protein (4E-BP1) and ribosomal S6 kinase (S6K) (Holz et al, 2005; Wullschleger et al, 2006). AMPK suppresses mTORC1 by two possible mechanisms. The first mechanism involves the phosphorylation of tuberous sclerosis 2 (TSC2), an upstream negative regulator of mTORC1 that has GTPase activator protein (GAP) activity against the small G protein Ras homolog-enriched in brain (Rheb). TSC2 is inhibited by Akt by phosphorylation on multiple serine residues. This phosphorylation has been shown to stimulate the

dissociation of TSC2 from Rheb at the lysosomal surface, with consequent conversion of Rheb to its active GTP-bound form (Menon et al, 2014). Phosphorylation by AMPK, at different sites, appears to activate TSC2 and thus inhibit Rheb; this results in inhibition of mTORC1 signalling (Inoki et al, 2003; Shaw, 2009). The second mechanism by which AMPK inhibits mTORC1, relies on the direct phosphorylation of Raptor, which leads to the binding of 14-3-3 proteinsto Raptor itself, and a consequent reduction in mTORC1 activity (Gwinn et al, 2008). Inhibition of mTORC1 results in the lack of phosphorylation of both 4E-BP1 and S6K. Suppression of their phosphorylation allows 4EBP1 to bind and inhibit the translational initiation factor eIF4E (Haghighat et al, 1995) and prevents S6K from upregulating the translation of certain mRNAs, such as those encoding hypoxia-inducible factor-1α, Myc and cyclin D1, all involved in cell cycle progression and cell growth (Guertin & Sabatini, 2007). The final outcome is reduced protein synthesis and cellular proliferation.

1.9.4 Regulation of cell cycle

DNA replication and mitosis are energy requiring processes and preventing their execution might be an additional mechanism to re-establish the energetic balance when the cell experiences stress conditions. AMPK causes arrest in G1 phase before DNA replication (Imamura et al, 2001). Arrest follows phosphorylation of p53 at Ser15. This site does not fit with AMPK recognition motif, which suggests that it might not be a direct phosphorylation. However, phosphorylation of Ser15 stabilizes p53 leading to the activation of a cyclin-dependent kinase inhibitor, $p21^{WAF1}$, that triggers G1:S phase cell cycle arrest (Imamura et al, 2001; Jones et al, 2005).

Phosphorylation of another cyclin-dependent kinase inhibitor, $p27^{KIP1}$, by AMPK also induces G1:S cell cycle arrest by stabilizing $p27^{KIP1}$. However, also in this case,

phosphorylation of p27KIP1 might not be direct, since the phosphorylation site at the C-terminal does not fit the AMPK consensus motif (Liang et al, 2007).

Unexpectedly, other studies suggest that AMPK activity is necessary for *completion* of mitosis. A chemical genetic screen identified several new AMPK targets that had roles in mitosis and cytokinesis (Vazquez-Martin et al, 2009). Among them, it is worth mentioning PPP1R12C, a regulatory subunit of PP1 phosphatase that targets PP1 to dephosphorylate myosin regulatory light chain (MRLC), and the protein kinase PAK2 that phosphorylates MRLC. Phosphorylation of PPP1R12C on Ser452 by AMPK, promote its binding with 14-3-3 proteins, thus preventing dephosphorylation of MRLC by PP1. Phosphorylation of MRLC seems to be crucial to the completion of mitosis (Banko et al, 2011). In mitotic cells phosphorylation of both AMPK and PPP1R12C is increased and mutation of Ser452 to alanine leads to an increase to multinucleated cells, which suggests a defect in mitosis. It is still not clear why AMPK activation promotes mitosis. One hypothesis could be that mitosis is accelerated so that cell cycle arrest can occur in the ensuing G1 phase. However it is not possible to rule out that this is an ancillary function of AMPK, not related to its role as an energy sensor.

1.9.5 Mitochondrial biogenesis and autophagy

AMPK has been demonstrated to promote mitochondrial biogenesis, a process that in the longer-term generates increased capacity for the oxidative catabolism of glucose, fatty acids and amino acids. Improved exercise endurance and increased expression of mitochondrial genes in muscles was observed in mice daily treated with AICAR (Winder et al, 2000). Mitochondrial biogenesis is regulated by the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α), which enhances the activity of a variety of transcription factors acting on nuclear-encoded mitochondrial genes (Lin et al,

2005). AMPK can promote mitochondrial biogenesis either by direct phosphorylation of PGC1α (Jager et al, 2007) or by promoting its deacetylation through an increase in the concentration of NAD^+, a co-substrate for the deacetylase sirtuin 1 (SIRT1) (Canto et al, 2010).

AMPK is also involved in the turnover of mitochondria through a specific kind of autophagy called mitophagy. Autophagy is a process regulating the degradation of damaged or dysfunctional cellular components to recycle important molecules such as lipids and amino acids. During autophagy, large vesicles, containing cytoplasmic components, fuse with lysosomes to form autophagosomes. Lysosomal hydrolases digest the content of autophagosomes, thus providing a source of amino acids and nutrients when they cannot be provided from somewhere else (Mizushima et al, 2008). Autophagosomal formation is initiated by UNC-51-like kinase 1 (ULK1) and ULK2, the mammalian orthologues of the yeast Atg1, which are phosphorylated and activated by AMPK (Egan et al, 2011). Phosphorylated ULK1 resulted in increased autophagy and mitophagy, where dysfunctional mitochondria are degraded. When cells lack functional ULK1 or when the AMPK phosphorylation site was mutated, mitochondria showed an aberrant morphology and an impaired function (Egan et al, 2011). AMPK regulation of both autophagy and mitophagy maintains the energy status of the cell under times of stress and maintains the health and functionality of mitochondria.

1.10 AMPK and human diseases

Having dozens of physiological targets, AMPK is a key regulator of energy balance at a cellular level as well as a whole-body level. It is then not surprising that in many human disorders, from inflammatory diseases to cancer, AMPK plays a significant role.

1.10.1 Type 2 diabetes

Type 2 diabetes is a systemic disease involving multiple pathophysiologic disturbances. It is a progressive condition that starts with a moderate- to severe insulin resistance in muscle and liver, impaired pancreatic β-cell glucose sensitivity and increased insulin secretion. With time, β-cells stop secreting sufficient amounts of insulin to offset insulin resistance, causing a condition called Impaired Glucose Tolerance, which then progresses to type 2 diabetes (Ferrannini & DeFronzo, 2015). Insulin resistance is associated with accumulation of triacylglycerols in liver and in skeletal muscle, especially in obese individuals whose capacity to store triacylglycerols in adipose tissue is exceeded. It has in fact been shown that individuals with lipodystrophy, who completely lack adipose tissue, store triacylglycerols in liver and muscle, which become insulin resistant (Samuel et al, 2010). Activated AMPK promotes the oxidation of fatty acids and inhibits the synthesis of new fatty acids and triacylglycerols. It also increases glucose uptake by skeletal muscle and prevents gluconeogenesis in the liver. Agents that activate AMPK are thus expected to reduce lipid stores in liver and skeletal muscle, improving insulin sensitivity, and to improve the hyperglycaemia associated with type 2 diabetes. Indeed treatment with AICAR reversed several metabolic abnormalities in animal models of obesity and insulin resistance (Buhl et al, 2002; Song et al, 2002). The direct AMPK activator, A769662, was also shown to increase fatty acid oxidation in rats, and to decrease plasma glucose, body weight gain, plasma and liver triacylglycerols and hepatic expression of gluconeogenic and lipogenic enzymes in *ob/ob* mice (Cool et al, 2006). Like A769662, the natural product berberine also improves glucose tolerance and reduces body weight in *db/db* mice (Lee et al, 2006). All the beneficial effects of metformin, currently the drug of choice for treatment of

type 2 diabetes, have been previously discussed in this chapter and, along with the other lines of evidence here reported, support the fundamental role that AMPK has in the progression of type 2 diabetes.

1.10.2 Cancer

The idea that AMPK could be involved in tumourigenic processes first arose when the tumour suppressor LKB1 was identified as the major upstream kinase phosphorylating AMPK (Hawley et al, 2003). As previously discussed in this chapter, individuals carrying mutations in the gene encoding LKB1 develop Peutz-Jeghers syndrome, a condition associated with the development of benign intestinal tumours, with an increased risk of developing malignant cancers at other sites (Nakau et al, 2002). Although there are 12 AMPK-related kinases phosphorylated by LKB1 that could potentially mediate its tumour suppressor effect, AMPK is the only one that has been proved to inhibit mTORC1, and almost all biosynthetic pathways required for cell growth, and to induce cell cycle arrest. Also, AMPK is known to promote oxidative metabolism, thus opposing the switch to aerobic glycolysis observed in many tumour cells (Vander Heiden et al, 2009). Consistent with this hypothesis, it has been shown that activators of AMPK, such as metformin, phenformin and A769662, delay tumour onset in tumour-prone mice (Huang et al, 2008). Furthermore, whole-body knockout of AMPK-α1 (the only isoform expressed in lymphocytes) increases the development of lymphomas in mice overexpressing Myc oncogene in B cells (Faubert et al, 2013). Several studies have also reported that patients treated with metformin had a lower incidence of cancer compared to those treated with a different medication (Evans et al, 2005). Although it is still not clear whether the beneficial effect of metformin on cancer development is entirely mediated by AMPK, there is evidence that treatment

with metformin or phenformin increases the rate of apoptosis in tumour cells that have lost LKB1 (Shackelford et al, 2013). However, the precise role of AMPK in cancer is still not completely understood. It has been shown that prolonged mitotic arrest causes a mitophagy-dependent loss of mitochondria, along with reduced ATP levels and subsequent AMPK activation. AMPK phosphorylates PFKFB3 (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3), which resulted in oxidative respiration to be replaced by glycolysis, suggesting a pro-cancer effect of AMPK (Domenech, E. et al, 2015). Also, a complete loss of AMPK function is rare in human cancers and when it occurred it resulted in a clear disadvantage in cancer cells survival, suggesting that, paradoxically, tumour cells need a certain level of AMPK for their proliferation (Hardie, D.G. 2015).

The growth-promoting protein kinase B/ Akt pathway is known to be hyperactivated in many human cancers. Hyper-activation is caused either by activating mutations in phosphatidylinositide 3-kinase (PI3K) or by loss-of-function mutations in the tumour suppressor PTEN (Yuan & Cantley, 2008). It has been reported that Akt phosphorylates rat AMPK-α1 on Ser485 and that this phosphorylation, inhibited the subsequent phosphorylation of Thr172 by LKB1 (Horman et al, 2006). Recently our laboratory has demonstrated that Akt can phosphorylate the equivalent site (Ser487) on human AMPK-α1 (Hawley et al, 2014). These sites lie in a loop termed the “ST-loop” which has been proved to interact with the C-helix on the AMPK kinase domain, blocking access of upstream kinases to Thr172. These data, along with the discovery that Ser487 is phosphorylated in three different human cell lines in which Akt is hyperactivated, suggest that Akt downregulates AMPK in tumour cells, underlying once more the possible importance of AMPK as a tumour suppressor.

1.10.3 Inflammatory disease

There is increasing evidence that the metabolic actions of AMPK may be involved in some anti-inflammatory effects (Steinberg et al, 2013). Unactivated dendritic cells, neutrophils and T-cells generate ATP usually through oxidative metabolism. However, once activated during an immune response, they switch to aerobic glycolysis, and it seems that this switch is coupled with a decrease in AMPK activation. Downregulation of AMPK has been shown to inhibit the switch to aerobic glycolysis while pharmacological activation of AMPK seems to promote it (Krawczyk et al, 2010). Studies performed in mouse models showed that AMPK-β1 knock-out in macrophages (β1 is the predominant form expressed in these cells) resulted in reduced ACC phosphorylation and mitochondrial content, as well as reduced rates of fatty acid oxidation that promoted the accumulation of pro-inflammatory diacylglycerols. In β1-deficient macrophages, A769662 also failed to stimulate fatty acid oxidation and, consequently, to suppress inflammation (Galic et al, 2011). These findings support the idea that AMPK mediates the anti-inflammatory response via its effects on fatty acid oxidation.

1.11 Experimental aims

AMPK is a crucial metabolic regulator and, as such, is involved in a variety of pathophysiological processes. Understanding the precise mechanisms by which it is activated by different molecules and compounds will give us additional tools to further investigate metabolic events, whose critical role in numerous diseases is now emerging. Oxidative stress, mediated by hydrogen peroxide, has been shown to activate AMPK. However the precise mechanism of activation as well as its possible biological meaning remained to be clarified. The aim of Chapter 3 of this thesis will be

to investigate whether AMPK is activated by hydrogen peroxide through a direct or an indirect mechanism of activation and to discuss the importance of such activation.

Furthermore, recent evidence provided new insights into the structure of AMPK, allowing the identification of the binding site where some direct activators exert their effect. The characterization of this novel binding pocket will be the focus of Chapter 4 of this thesis.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

Sodium fluoride, sodium pyrophosphate, Triton X-100, benzamidine hydrochloride, phenylmethylsulfonylfluoride (PMSF), soyabean trypsin inhibitor (SBTI), berberine chloride, A23187, sodium salicylate, phenformin, dimethyl sulfoxide (DMSO), hydrogen peroxide, SU6656, glucose oxidase, catalase, magnesium chloride, Serva blue G, sodium pyruvate, sodium ethylenediaminetetraacetate (EDTA), tri-n-octylamine and 1,1,2-trichlorotrifluoroethane were from Sigma (Poole, UK). Hepes, Tris(hydroxymethyl)methylamine (Tris), dithiothreitol (DTT) and isopropyl-α-D-thiogalactopyranoside (IPTG) were from Fomedium (Hunstanton, UK). Sodium ethylenebis(oxyethylenenitrilo)-tetraacetate (EGTA), sodium chloride, orthophosphoric acid, Brij-35, Tween-20, ethanol, methanol and 2-propanol were from VWR (UK). STO609 was from Tocris (UK). [γ-^{32}P]ATP was from Perkin Elmer (Bucks, UK). ATP, ADP and AMP were from Melford (CHelsworth, UK). EDTA-free protease cocktail inhibitor tablets were from Roche Diagnostics (Lewisham, UK). Protein G-Sepharose, 5 ml HisTrap FF columns, and HiLoad 16/60 Superdex 200 pg column were from GE Healthcare (Bucks, UK). Dulbecco's Modified Eagle's Medium (DMEM), McCoy's 5A medium, Foetal Bovine Serum (FBS), blasticidin, hygromycin B, trypsin-EDTA and penicillin-streptomycin solution (pen-strep) were from Life Technologies (UK). FuGENE was from Promega (UK). A769662 was manufactured as described previously (Iyengar et al., 2005). Compound 991 was kindly provided by Benoit Viollet (Institue Cochin, Paris).

2.1.2 Molecular biology reagents

QIAprep Spin Miniprep kit, QIAprep Hi-Speed Plasmid Maxi kit, QIAquick PCR purification kits and QIAquick Gel Extraction kit were from QIAGEN (Crawley, UK). QUikchange II site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Molecular grade agarose and dNTPs were from Sigma (Poole, UK). Blue/orange 6X loading dye was from Promega (Southampton, UK). XL-10 Gold and OneShot BL21 (DE3) and XL-1 competent *Escherichia coli* were from Life Technologies (UK). Liquid LB media and plates supplemented with ampicillin or kanamycin, SOC media and autoinduction media were supplied by Media Service, College of Life Sciences, University of Dundee.

2.1.3 Plasmids

Plasmids used in this thesis are shown in table 2.1 and were kindly provided by Dr. Fiona Ross, University of Dundee.

Plasmid	Vector	Expression	Tag
AMPK-α1	pCMV	Mammalian	FLAG
AMPK-α2	pCMV	Mammalian	FLAG
AMPK-α1β1γ1	pcDNA 5/FRT/TO	Bacterial	His
GST-CaMKKβ	pGEX	Bacterial	GST

Table 2.1. Plasmids used in this thesis

2.1.4 Primers

Primers used in this thesis were from Sigma and are given in table 2.2

Mutation	Forward primer	Reverse primer
AMPK-α1[K40A]	CACCTTCGGCGCAGTGAAG GTTGGCAAACATGAATTG	CAATTCATGTTTGCCAACCTTCA CTGCGCCGAAGGTG
AMPK-α1[K42A]	CCTTCGGCAAAGTGGCGGT TGGCAAACATGAATTG	CAATTCATGTTTGCCAACCGCC ACTTTGCCGAAGG
AMPK-α1[K40A/K42A]	CCTTCGGCGCAGTGGCGGT TGGCAAACATGAATTGACT GGG	CCCAGTCAATTCATGTTTGCCAA CCGCCACTGCGCCGAAGG
AMPK-α2[K29A]	CACCTTCGGCCGAGTGAAG ATTGGAGAACATCAATTAA CAG	CTGTTAATTGATGTTCTCCAATC TTCACTCGGCCGAAGGTG
AMPK-α2[K31A]	CACCTTCGGCAAAGTGGCG ATTGGAGAACATCAATTAA CAG	CTGTTAATTGATGTTCTCCAATC GCCACTTTGCCGAAGGTG
AMPK-α2[K29A/K31A]	CTTCGGCCGAGTGGCGATT GGAGAACATCAATTAAC	GTTAATTGATGTTCTCCAATCGC CACTCGGCCGAAG

Table2.2. Primers used in this thesis

Mutants created by site-directed mutagenesis are shown in table 2.3

Mutant	Plasmid DNA	Primers
AMPK-α1[K40A]β1γ1	pcDNA 5/FRT/TO	AMPK-α1[K40A]
AMPK-α1[K42A]β1γ1	pcDNA 5/FRT/TO	AMPK-α1[K42A]
AMPK-α1[K40A/K42A]β1γ1	pcDNA 5/FRT/TO	AMPK-α1[K40A/K42A]
AMPK-α2[K29A]	pcDNA 5/FRT/TO	AMPK-α2[K29A]
AMPK-α2[K31A]	pcDNA 5/FRT/TO	AMPK-α2[K31A]
AMPK-α2[K29A/K31A]	pcDNA 5/FRT/TO	AMPK-α2[K29A/K31A]

Table 2.3. AMPK mutants created as part of this thesis

2.1.5 Protein biochemistry reagents

XCell Surelock™ Mini-Cell, XCell Blot Module™, iBlot transfer module, NuPAGE® LDS Sample Buffer (4X), pre-cast NuPAGE Novex 4-12% Bis-Tris gels and 3-8% Tris-acetate gels, NuPAGE MOPS SDS running buffer (20X), SeeBlue Plus2 Pre-Stained Standard and IRDye 680 secondary antibodies were from Life Technologies (UK). IRDye800 secondary antibodies were from Rockland (Gilbertsville, PA). Bovine serum albumin (BSA) was from Sigma (Poole, UK). Vivaspin protein concentrators were from Sartorius (UK).

2.1.6 Proteins

Rat liver AMPK was purified as described previously (Hawley et al., 1996) except that the final size-exclusion chromatography step was on a Superdex 20 (Hi load 16/60) column, not on Sephacryl S-200. A GST-fusion of human CaMKKβ and His-fusion human AMPK α1β1γ1, AMPK α1[K40A]β1γ1, AMPK α1[K42A]β1γ1 and AMPK α1[K40A/K42A]β1γ1 constructs were expressed and purified from *E.coli*. PP2Cα was purified from *E.coli* by the Division of Signal Transduction Therapy (DSTT), University of Dundee.

2.1.7 Peptides

The AMARA and SAMS peptides were synthesised by GL Biochem (Shanghai, China) and are detailed in table 2.4

Peptide	Sequence	Reference
AMARA	AMARAASAAALARRR	(Dale et al, 1995)
SAMS	HMRSAMSGLHLVKRR	(Davies et al, 1989)

Table 2.4. Peptides used in this thesis.

2.1.8 Antibodies

Antibody	Species	Recognises	Company	Catalogue No.
Phospho AMPK-α (Thr172)	Rabbit	AMPK-α1 and α2 phosphorlated at Thr172	Cell Signalling	

AMPK α1	Rabbit	AMPK α1	Cell Signalling	
AMPK β1	Rabbit	AMPK β1	Abcam	
AMPK γ1	Rabbit	AMPK γ1	Abcam	
FLAG	Mouse	FLAG peptide (DYKDDDDK)	Sigma	
GAPDH	Mouse	GAPDH	Abcam	

Table 2.5. Commercial antibodies used in this thesis

Antibody	**Species**	**Immunogen**	**Recognizes**	**Reference**
AMPK-α1	Sheep	CTSPPDSFLDDHHLTR (344-358 of rat AMPK-α1)	AMPK-α1	Woods et al. 1996
AMPK-α2	Sheep	CMDDSAMHIPPGLKPH (352-366 of rat AMPK-α2)	AMPK-α2	Woods et al. 1996
Phospho ACC	Sheep	TMRPSMSGLHLVK (217-226 of human ACC2)	ACC1/ACC2 phosphorylated at Ser79/Ser221 respectively	Hawley et al. 2003

Table 2.6. Non-commercial antibodies used in this thesis

2.1.9 Buffers

Hepes assay buffer:

50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM DTT

IP Buffer (low salt): 50 mM Tris-HCl pH 7.25, 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine hydrochloride, 0.1 mM PMSF, 5 μg/ml soyabean trypsin inhibitor, 1 % (v/v) Triton-X100

IP buffer (high salt): 50 mM Tris-HCl pH 7.25, 500 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine hydrochloride, 0.1 mM PMSF, 5 μg/ml soyabean trypsin inhibitor, 1 % (v/v) Triton-X100

Lysis buffer (mammalian cells): 50 mM Tris-HCl pH 7.25, 150 mM NaCl, 50 mM NaF, 1 mM NaPPi, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine hydrochloride, 0.1 mM PMSF, 5 μg/ml soyabean trypsin inhibitor, 1 % (v/v) Triton-X100

Lysis buffer (bacterial cells): 50 mM Tris-HCl pH 8.1, 500 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mg/ml lysozime with Complete Protease Inhibitor mix

Elution buffer: 50 mM Hepes pH 8.0, 200 mM NaCl, 20 mM glutathione

TBS-tween: 20 mM Tris-HCl pH 7.4, 137 mM NaCl, 0.1 % Tween-20

Coomassie stain: 50 % (v/v) methanol, 10 % (v/v) acetic acid and 0.1 % (w/v) Coomassie Brilliant blue

Coomassie destain: 10 % (v/v) methanol, 10 % (v/v) acetic acid

Low imidazole buffer: 50 mM Tris-HCl pH 8.1, 500 mM NaCl, 20 mM Imidazole

High imidazole buffer: 50 mM Tris-HCl pH 8.1, 200 mM NaCl, 1 M Imidazole

Dialysis buffer: 50 mM Hepes pH 8.1, 200mM NaCl

Dialysis buffer (phosphorylated proteins): 50 mM Hepes pH 8.1, 200mM NaCl, 50 % (v/v) glycerol

6X Sample Buffer: 1.2 ml 4 x Tris 1M pH 6.8, 4.7 ml glycerol, 1.2 g SDS, 0.93 g DTT (0.6 M), 6 mg bromphenol blue, 2.1 ml H_2O.

2.1.10 Media

The media kitchen, University of Dundee, provided all bacterial culture media.

Bacterial culture medium: *Luria Bertani* broth: 10 g bacto-tryptone was added to 5 g bacto-yeast extract, 10 g NaCl, 950 ml double distilled and deionised water and adjusted to pH 7.0 with NaOH. The medium was autoclaved for 20 minutes at 15 psi then antibiotics were added to their final concentration. LB plates contained the above plus 15 g L^{-1} of bacto-agar.

SOC (Super Optimal Broth/Catabolite Repression): 30 g of bacto-tryptone was added to 5 g bacto-yeast extract, 0.5 g NaCl and dissolved. 10 ml KCl was added from a stock concentration of 250 mM and 5 ml of $MgCl_2$ from a stock concentration of 2 M. The medium was autoclaved for 20 minutes at 15 psi and allowed to cool. 20 ml glucose was added from a stock concentration of 2 M, the pH adjusted to 7.0 and made up to 1 L with double distilled and deionized water.

2.2 Methods

2.2.1 Site-directed mutagenesis

Site-directed mutagenesis on DNA constructs was performed using Statagene QuikChange II site-directed mutagenesis kit according to manufacturer's instructions. The mutagenesis reactions were set up in sterile 0.2 ml PCR tubes and each reaction contained: 1X reaction buffer, template plasmid dsDNA (50ng), forward and reverse mutagenic oligonucleotide mutagenesis primers (0.3 μM each), 1 mM dNTPs, 2.5 U *Pfu turbo* DNA polymerase made up to a final volume of 50 μl with sterile de-ionised water. The reactions were performed in a Hybrid PCR express thermal cycler using the following conditions: [95°C (1 min)] x 1, [95°C (1 min), 55°C (1 min), 68°C (1 min/kb plasmid length)] x 18, [68°C (10 min)] x1. After cycling, 10 U of *DpnI* digests methylated DNA (template plasmid DNA) but not non-methylated DNA (mutant plasmid). This reaction mixture(10 μl) was used to transform competent XL-10 Gold *E.coli* cells following the protocol explained in section 2.2.2 of this section. DNA constructs were verified by DNA sequencing.

2.2.2 Transformation of *E.coli*

Bacterial cells were defrosted in ice, 1 μl of the selected DNA was added and the cells were then incubated on ice for 30 minutes. The cells were subject to heat shock at 42°C for 45 seconds, before incubation on ice for 2 minutes. SOC medium (250 μl) was added to the cells prior to incubation in a shaking incubator at 37°C for 1 h. The cells were then streaked on an LB plate containing the appropriate antibiotic for selection by incubation at 37°C overnight.

2.2.3 Purification of plasmid DNA from *E.coli*

Small scale purification: Transformed bacterial cells were grown overnight at 37°C in 5 ml of LB broth supplemented with the appropriate antibiotic for selection. The cells were pelleted by centrifugation at 13000 rpm for 2 minutes. Plasmid DNA was purified using the QIAGEN QIAprep Spin Miniprep kit according to manufacturer's instructions. The cells were resuspended in buffer P1 and lysed in buffer P2 of the kit. The SDS contained in these buffers solubilizes proteins and phospholipid, leading to lysis of the cells and the alkaline conditions ensure denaturation of chromosomal/plasmid DNAs and protein. Buffer N3 of the kit was used to neutralize and adjust the lysates to high salt conditions. In this step chromosomal DNA, proteins and SDS precipitate while plasmid DNA remains in solution and is separated from the precipitate by centrifugation before being applied to a column containing a silica membrane. The membrane binds the plasmid DNA under high salt conditions. Salts were removed by washing with an ethanol-based buffer and plasmid DNA was eluted using sterile deionized water. DNA was sequenced as described below in section 2.2.5.

Large scale purification: *E.coli* cells were grown at 37°C overnight in 200 ml of LB broth plus the appropriate antibiotic for selection. The cells were centrifuged at 7000 rpm for 15 minutes at 4°C and the plasmid DNA was extracted using the QIAGEN Hi-Speed Plasmid Maxi kit according to manufacturer's instructions. As for small scale purification, the cells were resuspended in P1 and lysed in P2 buffer from the kit. The lysates were then neutralized with buffer P3, cleared by filtration and then applied to a column containing a silica membrane. DNA bound to the membrane was precipitated by the addition of isopropanol and collected using the QIAPrecipitator, before elution

in sterile deionized water. DNA was then sequenced as described below (section 2.2.5).

2.2.4 DNA quantification

1.5 µl of DNA was quantified using the NanoDrop 1000 Spectrophotometer from Thermo Scientific. Absorbance was measured at 260 nm, against a water blank, to give an estimation of the DNA present in the sample. For double-stranded DNA, an Optical Density (OD) of 1 at 260 nm corresponds to a DNA concentration of 50 ng/µl.

2.2.5 DNA sequencing

DNA sequencing was performed by The Sequencing Service, College of Life Sciences, University of Dundee, using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

2.2.6 Expression of human recombinant proteins in *E.coli*

GST-fusion proteins: BL21 (DE3) competent cells were transformed with plasmid DNA and streaked on plates containing the appropriate antibiotic. A single colony was selected to inoculate 10 ml of medium overnight at 37°C. Five ml from the overnight culture was used to inoculate 1 L of medium. The medium was shaken at 200 rpm and 37°C until the absorbance at 600 nm was between 0.6 and 0.8. The culture was then put on ice for 10 minutes and 1 mM isopropyl-β-D-galactopyranoside (IPTG) was added. The culture was shaken at 160 rpm overnight at 20°C. The cells were pelleted by centrifugation at 7000 rpm for 15 minutes at 4°C, frozen in liquid nitrogen and stored at -20°C prior to purification.

His-fusion proteins: The same technique described for GST-fusion proteins was performed using Auto Induction medium (AIM). AIM has been formulated to grow IPTG-inducible expression strains, initially without induction, and then to induce production of target protein automatically, usually near saturation at high cell density. A limited concentration of glucose is metabolized preferentially during growth, which prevents uptake of lactose until the glucose is depleted, usually in mid to late log phase. As the glucose is depleted, lactose can be taken up and converted by β-galactosidase to the inducer allolactose. Allolactose causes release of lac repressor from its specific binding sites on DNA and thereby induces expression of T7 RNA polymerase from the lacUV5 promoter and unblocks T7lac promoters, allowing expression of target proteins by T7 RNA polymerase.

2.2.7 Purification of GST-fusion proteins

Frozen pellets obtained as explained in section 2.2.6 were resuspended in lysis buffer and left in ice for 30 minutes. The lysate was then sonicated usually for 10 x 10 seconds at 75 % of maximum, depending on the volume of the lysate, and clarified by centrifugation at 30000 rpm for 30 minutes at 4°C. Supernatant was applied to a pre-equilibrated 5 ml glutathione-Sepharose column at a flow rate of 1 ml/min at 4°C. The column was subsequently washed with 10 column volumes of lysis buffer and the protein was eluted in the elution buffer described in section 2.1.9.

2.2.8 Purification of His-fusion proteins

Frozen pellets obtained as explained in section 2.2.6 were ground to a fine powder using a mortar and pestle in the presence of liquid nitrogen. The powder was resuspended in lysis buffer and clarified as explained in section 2.2.7. The supernatant

was applied to a pre-equilibrated 5 ml HisTrap FF column at a flow rate of 1 ml/min. The column was washed with 10 volumes of low imidazole buffer and the protein was eluted over an imidazole gradient (20 mM to 1 M). Upon an increase in the absorbance at 280 nm, the gradient was held until no further protein was eluted. Relevant fractions were pooled together, dialysed overnight in the dialysis buffer described in section 2.1.9 and then stored at -80°C.

2.2.9 Phosphorylation of α1β1γ1 AMPK by CaMKKβ

Small scale experiment

Purified bacterial AMPK (0.5 μg) was incubated with increasing concentrations of CaMKKβ for 30 minutes with ATP (200 μM) and $MgCl_2$ (5 mM). AMPK activity was measured by kinase assay as described in section 2.2.24. The small scale experiment allowed the ratio between bacterial AMPK and CaMKKβ required for AMPK activation to be determined.

Large scale experiment

5-6 mg of bacterially expressed AMPK was incubated with CaMKKβ for 30 minutes at 30°C in presence of ATP (200 μM) and $MgCl_2$ (5 mM). The mixture reaction was then applied to a pre-equilibrated HiLoad Superdex 200 16/600 column, connected to a 5 ml glutathione-Sepharose column to retain GST-CaMKKβ. Purified fractions were eluted at a flow rate of 1-1.4 ml/min. Fractions containing phosphorylated AMPK were concentrated, dialysed against a glycerol-containing buffer and stored at -20°C.

2.2.10 General mammalian tissue culture

All media and buffers used for tissue culture were warmed to 37°C prior to use. Cells were cultured and maintained in 75 cm^3 or 175 cm^3 flasks at 37°C in an atmosphere containing 5% CO_2. The cells were grown until 80-90% confluency before splitting for routine maintenance. For passaging of cells, the culture medium was aspirated, 5-10 ml of trypsin-EDTA was added and the cells were returned to the 37°C incubator for 3-5 minutes. After the cells had detached from the surface of the flask, 1 ml of the cell suspension was used to seed a fresh 75 cm^3 or 175 cm^3 flask containing 15 ml or 25 ml of complete culture medium.

2.2.11 Freezing and thawing cell lines

Cells were grown to confluency in 175 cm^3 flasks and trypsinised as described in section 2.2.10. Cells were pelleted by centrifugation at 1000 rpm for 5 minutes, the trypsin was aspirated off and the cells were resuspended in growth media containing 10% (v/v) DMSO. The cells were transferred to cryo-protective tubes and frozen in a -80°C freezer before being transferred to liquid nitrogen for long-term storage. Frozen cell stocks were thawed in a 37°C water bath and transferred to 10 ml of pre-warmed media. Cells were pelleted and the supernatant was aspirated to remove DMSO. The cells were resuspended in fresh growth media and seeded into 75 cm^3 flasks.

2.2.12 HEK-293 cells

HEK-293 cells were cultured in DMEM supplemented with 10% (v/v) foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. HEK-293 stably expressing AMPK-γ2-FLAG, both WT and RG mutant, were kind gifts from Dr Fiona Ross, University of Dundee and cultured as above except that the medium was

supplemented with hygromycin B (200 μg/ml) and blasticidin (15 μg/ml). HEK-293 cells were generated using the Flp-In system to stably express AMPK-γ2-FLAG under the control of a tetracycline repressor. In the absence of tetracycline, the tet repressor (constitutively expressed from a gene stably inserted into the host genome) forms a homodimer that binds to a tet operator sequence in the promoter of a the gene of interest (in this case AMPK-γ2-FLAG) which had also been stably inserted to the genome. This represses transcription of the gene of interest. Upon addition, tetracycline binds to the tet repressor, rendering it unable to bind to the tet operator and allowing transcription of the gene of interest. Tetracycline (1 μg/ml) was added to the cells for 36 hr prior to the treatment and lysis. AMPK-$\alpha1^{-/-}$-$\alpha2^{-/-}$ HEK-293 cells, containing a Flippase Recognition Target (FRT) site incorporated using Flp-In technology according to the manufacturer's instructions, were a kind gift of Dr. Alex Gray and were cultured as standard HEK-293 cells.

2.2.13 HeLa cells

HeLa cells were cultured in DMEM supplemented with 10% (v/v) foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.2.14 G361 human melanoma cells

G361 cells were cultured in McCoys 5A media supplemented with 10% (v/v) foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

2.2.15 Generation of HEK-293 cells expressing wild-type, K29A, K31A and K29A/K31A AMPK-α2

These cells were generated using Flp-In technology according to the manufacturer's instructions. A construct encoding human AMPK-α2 fused to an N-terminal FLAG tag

was inserted into the pcDNA/FRT plasmid. FRT-containing AMPK-$\alpha1^{-/-}$-$\alpha2^{-/-}$ HEK-293 cells were transfected with AMPK-α2 plasmid and a plasmid (pOG44) encoding a Flp recombinase. Transfection was performed using FuGENE according to manufacturer's instructions. After 48 hr, hygromycin B (100 μg/ml) and blastacydin (15 μg/ml) were added and the medium changed every 4-5 days until single clones could be identified and expanded. The same procedure was also performed using the following constructs: (i) human AMPK-α2 [K29A]; (ii) human AMPK-α2 [K31A]; (iii) human AMPK-α2 [K29A/K31A]. All the constructs were fused to an N-terminal FLAG tag.

2.2.16 Lysis of mammalian cells

Cells were harvested for rapid lysis, using a method which minimizes activation of AMPK. Dishes were placed on ice and the media discarded. The cells were washed with ice-cold PBS, lysed in 200 μl lysis buffer and scraped into pre-chilled 1.5 ml tubes. Lysates were clarified by centrifugation for 10 minutes at 12000 rpm, at 4°C. The supernatant was collected and the protein concentration determined. Lysates were flash frozen in liquid nitrogen and stored at -80°C. Prior to use, lysates were thawed on ice.

2.2.17 Estimation of protein concentration

Protein concentrations were estimated using Bradford reagent. A standard curve was generated using a range of BSA concentrations. Protein concentrations of samples were determined by adding 200 μl of Bradford reagent to 1 μl of protein sample, and measuring the absorbance at 595 nm.

2.2.18 SDS-PAGE

SDS-PAGE separates proteins according to molecular weight. The anionic detergents sodium or lithium dodecyl sulphate (SDS or LDS) are used to denature proteins and confer a negative charge proportional to their size resulting in a constant mass/charge ratio. When subject to an electric field, proteins migrate towards the anode at a rate proportional to their molecular weight.

Samples to be resolved were denatured by the addition of 6X sample buffer and heated at 70°C for 10 minutes. Samples, along with pre-stained molecular weight standards (Invitrogen SeeBlue Plus 2), were loaded onto pre-cast NuPAGE 4-12 % bis-tris gels and subject to electrophoresis at 150 V for about 1 hr in NuPAGE 1X MOPS running buffer. For separation of acetyl-Co-A carboxylase (ACC), pre-cast 3-8 % tris-acetate polyacrylamide gels were used and for electrophoresis at 150 V for 80 minutes in NuPAGE 1X tris-acetate running buffer after which proteins were transferred to nitrocellulose membranes for immunoblotting or stained with Coomassie Brilliant Blue for visualization of protein bands.

2.2.19 Coomassie Blue staining of gels

To visualize proteins after SDS-PAGE separation, gels were soaked for 30 minutes in Coomassie Brilliant Blue staining solution. Gels were then washed in Coomassie destain solution overnight. All incubations were performed at room temperature on a rocker platform. Imaging was performed using the LiCor Odyssey detection system (700 nm channel).

2.2.20 Immunoblotting (Western blotting)

Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblotting. For gels consisting of up to 15 wells, transfers were performed using an Invitrogen XCell II Blot Module according to manufacturer's instructions. The gel membrane sandwich was assembled as follows: 2x blotting pad, 3 MM filter paper, gel, nitrocellulose membrane, 3MM filter paper, blotting pads. The sandwich was orientated to place the gel closest to the cathode and the membrane closest to the anode. Transfers were performed at 35 V for 90 minutes using 1X NuPAGE transfer buffer containing 20 % (v/v) methanol.

For gels containing more than 15 wells, transfers were performed using the Invitrogen iBlot dry blotting system according to manufacturer's instructions. The gel-membrane sandwich was prepared as follows: anode stack (containing nitrocellulose membrane), gel, 3 MM filter paper, cathode stack, sponge. Transfers were performed at 20V for 7 minutes. Membranes were blocked in LiCor Odyssey blocking buffer for 1 hr then probed with primary antibody overnight. Membranes were washed 3 x 10 minutes with TBST buffer. Membranes were then incubated with the appropriate IRDye 680 or IRDye 800 secondary antibodies diluted 1:5000 in TBST for 45 minutes. Membranes were washed as above and the signal detected using the LiCor Odyssey IR detection system.

2.2.21 Non covalent-coupling of antibodies to protein G-sepharose beads

Protein G-sepharose beads (stored in 20 % ethanol) were washed four times with low-salt IP buffer to remove alcohol. The beads were then incubated with the required antibody (1 μg of antibody/ μl of beads) on a roller mixer at 2-8°C overnight to allow

coupling of antibody to beads. The beads were washed three times in high-salt IP buffer to remove unbound antibody and then with low-salt IP buffer for storage as a 30 % (v/v) slurry at 2-8°C until required.

2.2.22 Immunoprecipitation and assay of AMPK from cell lysates

Anti AMPK-α1 and α2 antibodies were non-covalently coupled to protein G-sepharose beads as described in section 2.2.21. The beads were then incubated with cell lysates on a roller mixer for 2 hr at 2-8°C to immunoprecipitate AMPK. When AMPK presented an N-terminal FLAG tag, immunoprecipitation was performed using EZview™ Red ANTI-FLAG® M2 Affinity Gel. The beads were washed 1X with high-salt IP buffer, 1X with low-salt IP buffer and 1X with Hepes assay buffer. The beads were then resuspended in Hepes assay buffer and aliquoted in 1.5 ml tubes in a final volume of 20 μl for subsequent kinase assay.

The activity of AMPK was measured by its ability to phosphorylate the synthetic AMARA peptide (AMARAASAAALARRR), which was derived from the sequence of rat acetyl-coenzyme A carboxylase (ACC), a substrate of AMPK (Dale et al., 1995). Assay mixture were started by the addition of 30 μl of assay buffer, containing 200 μM AMP, 200 μM [γ^{32}P]ATP, 5 mM $MgCl_2$ and 200 μM AMARA, in a final volume of 50 μl. Assays were incubated at 30°C in an orbital shaker for 15 minutes. Reactions were terminated by pipetting 30 μl of assay mixture onto squares of P81 phosphocellulose paper and placing these into a solution of 1 % (v/v) orthophosphoric acid. The papers were washed with water to remove any unincorporated ATP and allowed to dry at room temperature. The radioactivity incorporated in each sample was measured using an LKB-Wallace 1214-Rackbeta scintillation counter. One unit of activity is the amount of kinase catalyses the incorporation of one nmol of ^{32}P into the synthetic peptide.

2.2.23 Allosteric stimulation of AMPK immunoprecipitated from cell lysates

Immunoprecipitations and assays were carried out as described in section 2.2.22 but SAMS peptide was used in place of AMARA and the kinase reactions were performed in presence or absence of allosteric ligands (i.e. 200 μM AMP, 10 μM A769662).

2.2.24 AMPK assay ofbacterially expressed AMPK heterotrimers

AMPK assays were performed using either the SAMS or the AMARA peptide (200 μM) and a reaction mixture containing AMP (200 μM, unless otherwise indicated in the figure legends), ATP (200 μM, unless otherwise indicated in figure legends) and $MgCl_2$ (a constant 4.8 mM molar excess over ATP was maintained). The final volume of the reactions was 25 μl and incubation was at 30°C for 15 minutes after which 15 μl aliquots of the assay mixture were spotted onto squares of P81 filter paper for stopping in 1% (v/v) orthophosphoric acid. The incorporation of radioactivity was measured as described in section 2.2.22.

2.2.25 Protection against dephosphorylationin in-solution assays

Bacterially expressed AMPK, phosphorylated by CaMKKβ as described in section 2.2.9, was incubated with enough PP2Cα to give approximately 70 % Thr172 dephosphorylation. AMP, A769662 and salicylate were added at the concentrations indicated in the figures. Reactions were started by addition of $MgCl_2$ and stopped by diluting the reaction in Hepes assay buffer. Aliquots were removed for kinase assays as described in section 2.2.24.

2.2.26 Protection against dephosphorylation using immunoprecipitated AMPK

Immunoprecipitation was carried out as described previously. After the last wash in Hepes buffer, the beads were resuspended in Hepes buffer and aliquoted in 1.5 mL Eppendorf tubes (100 μL aliquots). 70 μL of the supernatant was removed and in each Eppendorf tube 5 μL of phosphatase (PP2C) was added. As a negative control 5 μL of Hepes were added. To start the reaction, 10 μL of a 1:1 mixture of 50 mM ATP and 98 mM $MgCl_2$ was added to each tube. The reaction was carried out for 10 minutes at 30˚C and then stopped by adding 1 mL of Hepes buffer. The tubes were spinned, the supernatant discarded and an additional 1 mL Hepes wash was performed. Samples were recentrifuged, the supernatant discarded and the beads were resuspended in Hepes and aliquoted into new tubes (100 μL). 80 μL of supernatant were discarded. AMPK assays were performed as described previously using AMARA peptide and the kinase reactions were carried out in the presence or absence of ligands (i.e. 200 μM AMP, 10 μM A769662, 3 μM 991, 100 μM MT63-78, 10 mM salicylate).

2.2.27 Nucleotide measurements

Culture medium was removed from cells, which were then washed in ice-cold PBS and lysed in ice-cold 5 % (v/v) perchloric acid. The samples were vortexed and clarified by centrifugation at 12000 rpm, 4°C for 10 minutes. The supernatant was collected and an equal volume of 1:1 mixture of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane added. The samples were vortexed, centrifuged at 12000 rpm, 4°C for 3 minutes and the top aqueous phase collected. This procedure was repeated twice more and the pooled acqueous phases were stored at -20°C before analysis.

Sample analysis was performed using a Beckman Coulter P/ACE 5500 capillary electrophoresis instrument using 50 mM sodium phosphate, 50 mM NaCl (pH 5.2, leading buffer) and 100 mM MES/Tris (pH 5.2, tailing buffer). Each buffer contained 0.2 % (v/v) hydroxymethylcellulose. Nucleotide peaks were detected by UV absorbance at 254 nm and nucleotide ratios were calculated using peak heights. Retention times for each nucleotide were determined prior to sample analysis by running a mixture of pure nucleotides through the capillary.

2.2.28 Hydrogen peroxide measurement in cell medium

Cells medium was collected and centrifuged at 10000 rpm for 5 minutes to remove insoluble particles. The supernatant was then assayed with the Cell Biolabs' OxiSelect^{TM} Hydrogen Peroxide Assay Kit. The OxiSelect™ Hydrogen Peroxide Assay Kit is a quantitative assay for measuring hydrogen peroxide in aqueous and lipid samples. For aqueous samples, sorbitol first converts peroxide to a peroxyl radical, which oxidizes Fe^{2+} into Fe^{3+}. For lipid samples, peroxide converts Fe^{2+} into Fe^{3+} directly. Then Fe^{3+} reacts with an equal molar amount of xylenol orange in the presence of acid to create a purple product that absorbs maximally between 540-600 nm. The antioxidant BHT is provided to prevent further undesirable chain peroxidation. The peroxide content in unknown samples is determined by comparison with the predetermined H_2O_2 standard curve.

2.2.29 Data analysis

Data, unless indicated otherwise, is mean ± SEM. Unless otherwise stated, statistical analysis was by ANOVA, using Bonferroni's multiple comparison test of selected data sets (*P<0.05, **P<0.01, ***P<0.001, P ≤ 0.0001).

Chapter 3: AMPK is activated by oxidative stress mainly through increases in cellular AMP/ATP ratios

3.1 Introduction

3.1.1 Oxidative stress

Reactive oxygen species (ROS) are by-products of aerobic metabolism, mostly originating in mitochondria. They consist of free radical and non-radical species, derived from the partial reduction of oxygen (Ray et al, 2012). In aerobic conditions 90% of the oxygen consumed is reduced to water by cytochrome oxidase through a four-electron mechanism in the electron-transport chain (ETC) (Ott et al, 2007). The electron transport chain is located in the inner mitochondrial membrane and its activity is coupled with pumping of H^+ out of the membrane. The resulting H^+ gradient is then used to drive synthesis of ATP from ADP, thus conserving some of the energy dissipated in the form of a high ratio of ATP to ADP. The remaining 10% of molecular oxygen undergoes a one-electron reduction forming the superoxide anion free radical ($O_2^{\cdot -}$). The superoxide anion is considered the "primary" ROS and can interact with other molecules to form "secondary" ROS (Valko et al, 2005). A further one-electron reduction of the superoxide anion, along with the addition of 2 protons, leads to the formation of the secondary ROS, hydrogen peroxide (H_2O_2). The superoxide anion can be converted into H_2O_2 either spontaneously, or enzymatically through the catalytic activity of superoxide dismutases (SOD), enzymes that are located in the cytosol and mitochondrial membrane (SOD1), and also in the mitochondrial matrix (SOD2). Superoxide dismutases prevent the accumulation of the superoxide anion under physiological conditions (Schieber & Chandel, 2014). H_2O_2 is also generated by the activity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases

(Lambeth, 2004). The third and most reactive ROS is the hydroxyl radical (˚OH), which originates from H_2O_2 in presence of ferrous ions, via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{®}OH + OH^-$) (Pastor et al, 2000). At high concentrations, ROS can induce damage to cell structures by reacting with nucleic acids, lipids and proteins. For example the hydroxyl radical is able to react with the DNA, damaging the purine and the pyrimidine bases, as well as the deoxyribose phosphate backbone (Dizdaroglu & Jaruga, 2012). This kind of oxidative damage is thought to represent an early step in mutagenesis, carcinogenesis and ageing. Polyunsaturated fatty acid residues within phospholipids are also extremely susceptible to oxidation by ROS (Siems et al, 1995). The major products of lipid peroxidation are malondialdehyde and 4-hydroxy-2-nonenal, the first being mutagenic and carcinogenic, while the second is extremely toxic (Wang et al, 1996). The side chains of amino acids within protein, especially cysteine and methionine residues, are also a well-known targets of ROS action (Stadtman, 2004). The possibility of being exposed to different forms of damage caused by ROS has led living organisms to develop a variety of defence mechanisms. Antioxidant systems are aimed either at eliminating ROS, minimizing of their deleterious effects, and they can be placed essentially in two main groups, i.e. low molecular mass antioxidants and high molecular mass antioxidants (Lushchak, 2014). A series of different compounds belong to the first group, such as vitamins C (ascorbic acid) and E (α-tocopherol), carotenoids, anthocyanins, polyphenols and uric acid. Almost all of these antioxidants are taken up by humans either by ingestion of natural foodstuffs, or as supplements. However, there is another low molecular mass antioxidant that is synthesized by most living organisms: the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine or GSH). GSH controls ROS levels in different ways: (i) it scavenges the hydroxyl radical directly; (ii) it regenerates vitamins C and E to their active forms, reducing the tocopherol radical

directly or reducing semihydroascorbate to ascorbate (Pastore et al, 2003); (iii) it is a cofactor for detoxifying enzymes (Lushchak, 2012). These detoxifying enzymes belong to the second group of antioxidant systems, and include the superoxide dismutases (SOD), glutathione peroxidase (GPx), and catalase. The concentration of ROS in the cell is generally kept low through the balance between the rate of their production and the rate of their elimination by these antioxidant systems. However there are several events that can disturb this equilibrium, such as: (i) depletion of reserves of low molecular antioxidants; (ii) a decrease in the production or inactivation of antioxidant enzymes; (iii) increased level of compounds whose autoxidation is coupled with increased ROS production. The possible consequences of this disequilibrium depends on the level and location of ROS generation, the efficiency of the antioxidant defences and availability of energetic resources, as well as the cellular targets with which ROS interact. Under normal conditions, ROS levels can fluctuate within a certain range, so that a slight increase in their production (or a slight decrease in antioxidant systems) does not have deleterious effects, and may even be necessary for certain physiological functions. There are a great number of such physiological functions that are indeed controlled by redox-responsive signalling pathways, such as: (i) redox-regulated nitric oxide (NO) production; (ii) ROS production by NAD(P)H oxidase in phagocytic cells; (iii) regulation of vascular tone by NO; (iv) redox regulation of cell adhesion; (v)redox regulation of immune responses; (vi) ROS-induced apoptosis. However, when the balance between ROS production and elimination is disturbed more severely, ROS become mediators of the process known as oxidative stress (Betteridge, 2000). Under conditions of oxidative stress, cells usually block general programs such as growth and progress through the cell cycle, in order to develop responses aimed at neutralizing the negative effects of ROS. Oxidative stress has been correlated with several pathological

conditions which can be categorised into two main groups: (i) diseases characterised by conditions of mitochondrial oxidative stress; (ii) diseases characterised by inflammatory oxidative conditions and enhanced activity of enzymes such as NAD(P)H oxidase (Valko et al, 2007). A significant redox imbalance has also been found in various cancer cells suggesting possible correlations between oncogenesis and oxidative stress. Moreover, ROS-induced DNA damage can result in the arrest or the induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Marnett, 2000). Increased amounts of $O_2\bullet^-$ and H_2O_2 have been found in hypertensive patients, establishing the correlation between ROS-induced oxidative stress and the pathogenesis of hypertension (Romero & Reckelhoff, 1999). It has also been proposed that one of the major causes of the diabetic complications caused by hyperglycaemia could be oxidative stress. Hyperglycaemia stimulates ROS formation through oxidative phosphorylation, glucose auto-oxidation, NAD(P)H oxidase, lipooxygenase, cytochrome P450 monooxygenases and nitric oxide synthase (Brownlee & Cerami, 1981). Furthermore, the brain is extremely vulnerable to oxidative damage because of its high oxygen utilization, its content of oxidizable polyunsaturated fatty acids, and the presence of redox-active metals such as copper and iron. In this organ oxidative stress can be considered as a major causative factor in several neurodegenerative diseases. For example, the brain of patients with Alzheimer's disease shows a significant extent of oxidative damage associated with accumulation of amyloid-β peptide, the main constituent of amyloids deposits characterizing this disease (Butterfield et al, 2002).

3.1.2 Hydrogen peroxide

Hydrogen peroxide was firstly isolated by Thérnard in 1818. It has been clear for many years that high concentrations of this compound damage key cellular molecules such as lipids and DNA (Plaine, 1955). Given high enough concentrations and long enough reaction times, nearly every protein will react in some fashion with H_2O_2. In order to maintain a normal redox function, cells therefore need to carefully control H_2O_2 concentration (Makino et al, 2004). Peroxidases reduce H_2O_2 to water and at the same time oxidize a secondary reductant, such as GSH or ascorbate. Catalases instead utilize H_2O_2 itself as a reductant, oxidising it to molecular oxygen. A large class of peroxidases use a deprotonated cysteine thiol (or thiolate) as the reactive moiety. Their active site cysteine residue results in a deprotonated thiolate anion ($R\text{-}S^-$), which promotes a 10- to 100-fold more rapid reaction with H_2O_2. H_2O_2 plays a crucial role in oxidative stress, being the most stable ROS under physiological conditions (Davies, 1995). Upon exposure to increasing levels of H_2O_2, eukaryotic cells begin to oxidize their pools of free thiols with almost no alteration to the reduced glutathione pool and the overall reductive potential of the cell. This state is generally defined as mild oxidative stress (Halvey et al, 2005). This becomes severe oxidative stress when a further increase in H_2O_2 levels eventually affects the glutathione pool and the reductive potential of the cell. However there is increasing evidence that H_2O_2 is not only a mediator of oxidative stress, but also an intracellular signal. Interestingly, when its intracellular concentration is below 0.7 μM, H_2O_2 acts a signalling molecule, involved in the regulation of several biological and physiological processes (Stone & Yang, 2006). One of the mechanisms of redox signalling involves the oxidation of cysteine residues within proteins (Rhee, 2006). H_2O_2 oxidizes the thiolate anion ($Cys\text{-}S^-$) to the sulfenic

form (Cys-SOH), causing changes in the protein and altering its function. This reaction is reversible, and the sulfenic form can be reduced back to the thiolate anion again by the reductases thioredoxin (Trx) and glutaredoxin (Grx) so that the functions of the protein are restored. However, if the level of H_2O_2 further increases, the sulfenic form can become oxidized to sulfinic (SO_2H) or sulfonic (SO_3H) forms. These modifications are irreversible, resulting in permanent protein damage (Schieber & Chandel, 2014). Growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factors (FGF) or vascular endothelial growth factor (VEGF) use H_2O_2 to induce downstream effects on tyrosine phosphorylation (Bae et al, 1997; Lo & Cruz, 1995; Sundaresan et al, 1995; Ushio-Fukai et al, 2002). Many tyrosine phosphatases can be inactivated by H_2O_2 oxidation, while growth factor receptor tyrosine kinases show an increased activity after the oxidation of a critical active site cysteine (Paulsen et al, 2012). The serine/threonine MAP kinases Erk1/2, JNK and p38 as well as the protein kinase B (PKB or Akt) are activated by H_2O_2 which also modulates the duration of their signalling by inactivating the upstream components of their pathways (Truong & Carroll, 2013; Ushio-Fukai et al, 1999). Extensive evidence also links H_2O_2 with modulation of gene expression through regulation of several transcription factors, such as NF-κB (suggesting an involvement in the inflammation process), hypoxia-inducible factor-1α (HIF 1α), Nrf2, p53 and activator protein-1 (AP-1) (Covas et al, 2013; Groeger et al, 2009).

3.1.3 AMPK and hydrogen peroxide: previous studies

AMPK and H_2O_2 are involved in a variety of signalling pathways, modulating a series of different fundamental cellular processes. H_2O_2, being an important mediator of oxidative stress as well as a second messenger of signalling pathways, is likely to affect

cellular energy balance, while AMPK is a known sensor of the cellular energetic state. One might wonder whether there is a link between H_2O_2 and AMPK and, if so, how they might affect one another. Choi et al (2001) first proposed that increased levels of H_2O_2 were accompanied by AMPK activation, and suggested that this activation was indirect since it correlated with decreased levels of ATP. However, in 2009 it was reported that low levels of hypoxia, which are known to generate ROS, induced AMPK activation without apparent changes in the level of cellular nucleotides (Emerling et al, 2009). These authors also showed that pre-treatment of cells with anti-oxidants was not associated with an alteration in the AMP:ATP ratio. By contrast, in our laboratory HEK-293 cells stably expressing the RG mutant of the AMPK-γ2 subunit, which is insensitive to changes in adenine nucleotide levels, completely lost AMPK activation or increased Thr172 phosphorylation induced by treatment with H_2O_2, which suggested instead that AMPK activation by H_2O_2 was entirely dependent on changes in the AMP:ATP ratio (Hawley et al, 2010). A further level of uncertainty regarding the precise mechanism by which H_2O_2 activates AMPK was added when Zmijewski et al (2010) proposed a mechanism involving direct oxidation of Cys-299 and Cys-304 in the α1 subunit of AMPK, which appeared to be sufficient for AMPK activation. They therefore suggested an AMP:ATP-independent mechanism of activation. It is important to note that in primary cardiomyocytes an inhibitory effect of H_2O_2 on AMPK activation has also been claimed (He et al, 2014; Shao et al, 2014), thus leaving the precise nature of the H_2O_2-AMPK interaction still open to debate. The aim of this chapter will be to take advantage of well-established methodologies from our laboratory combined with new techniques presented in other studies, to try to address whether AMPK activation by the reactive oxygen species H_2O_2 occurs because of a direct oxidation of AMPK or through an indirect AMP:ATP dependent mechanism.

3.2 Results

3.2.1 AMPK activation correlated with changes in cellular nucleotides when H_2O_2 was generated using glucose oxidase

In our laboratory, the effect of H_2O_2 on AMPK had been previously studied using cultured HEK-293 cells stably expressing either the WT γ2 subunit of AMPK (WT cells) or its R531G mutant (RG cells), which renders AMPK insensitive to changes in cellular nucleotides. H_2O_2 had been added to these cells as a single bolus and AMPK activation measured 1 hour later, a time point used for all the other AMPK-activating agents tested in the study of Hawley et al (2010). However, Zmijewski and colleagues used the enzyme glucose oxidase to generate H_2O_2. Glucose oxidase, when added to the cell medium, catalyses the oxidation of some of the glucose present in the medium to H_2O_2 and D-glucono-δ-lactone, resulting in a slow but constant production of H_2O_2 and thus potentially representing a better model for physiological oxidative stress. Since it had not been studied previously, the first thing we decided to check was how much H_2O_2 was produced in HEK-293 cell medium following the incubation with glucose oxidase.

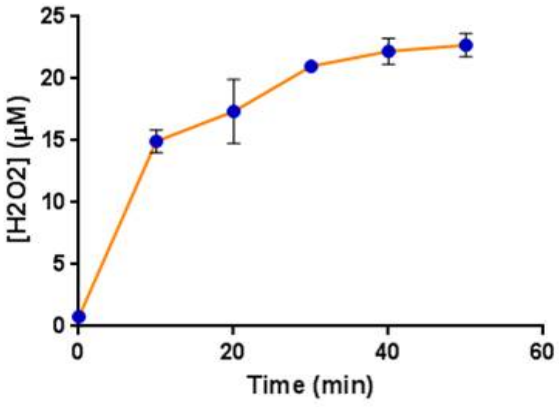

Figure 3.1. Measurement of H_2O_2 concentration after incubation with glucose oxidase. HEK-293 cells were incubated with 10 mU/ml glucose oxidase for increasing times. Cell media were collected and assayed for H_2O_2 content using the OxiSelect™ Hydrogen Peroxide Assay Kit (Colorimetric). Data represent the mean of 3 biological replicates ± SEM.

As shown in Figure 3.1, H_2O_2 concentration increased rapidly up to 20 minutes and then appeared to approach a plateau at around 20 μM.

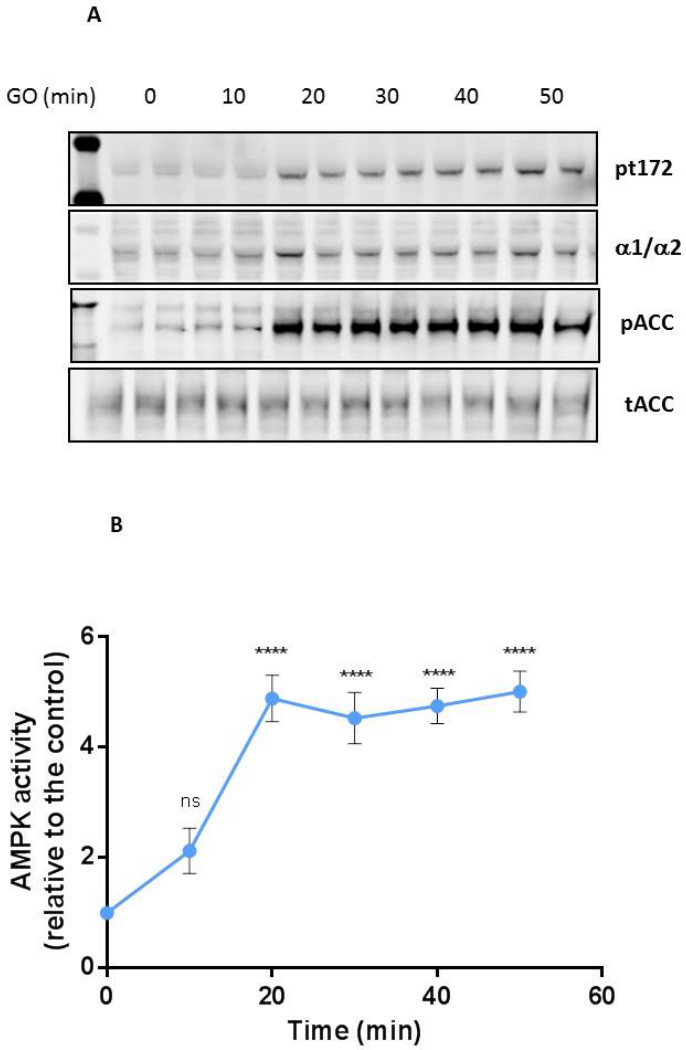

Figure 3.2. AMPK activation by incubation with glucose oxidase. HEK-293 cells were incubated with 10 mU/ml glucose oxidase for increasing times. **(A)** Western blot analysis to evaluate AMPK phosphorylation using anti-pT172 antibody and phosphorylation of the downstream target ACC at the AMPK site. Anti-α1/α2 and anti-ACC antibodies were used as controls for the total protein amounts. **(B)** AMPK from cell lysates was immunoprecipitated with α1/α2 antibodies and its activity assessed by peptide kinase assay. The normalized value of 1 corresponds to a basal activity of 0.3 nmol/min/mg. The graph represents the average of 3 independent experiments and the error bars represent the standard error of the mean (SEM). **** = $P \leq 0.0001$.

As suggested previously by Stone and Yang (2006), such extracellular concentrations results in an intracellular concentration of about 2 μM of H_2O_2, which is representative of a mild oxidative stress. We next wanted to assess the effect of exposure to glucose oxidase on AMPK activity Figure 3.2).As also observed by Zmijewski et al (2010), maximal phosphorylation of Thr172 was detected after 20 minutes of incubation with glucose oxidase, correlating with an increased phosphorylation of the downstream target acetyl-CoA carboxylase (ACC) (Figure 3.2A). Increased AMPK activity was also detected by kinase assays as shown in Figure 3.2B. However, when we measured the cellular nucleotide content after treatment with glucose oxidase, what we observed (Figure 3.3) significantly diverged from Zmijewski's findings.

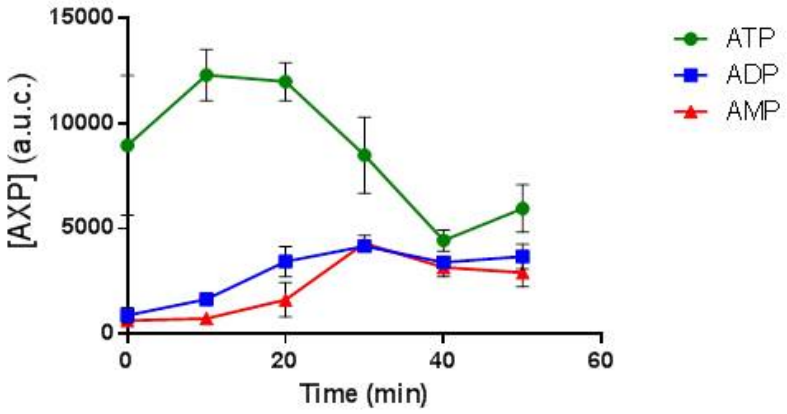

Figure 3.3 Estimated contents of ATP, ADP and AMP at various times after addition of glucose oxidase. HEK-293 cells were treated with glucose oxidase for increasing times. Cellular nucleotides were then extracted as explained in Materials and Methods and ADP and ATP content were measured by capillary electrophoresis. Values are area under curve (a.u.c.) of absorbance at 254 nm. Data represent 3 biological replicates ±SEM.

Figure 3.3 clearly shows that incubation of HEK-293 cells with glucose oxidase markedly modified the content of cellular adenine nucleotides. There were decreases

in ATP and increases in both AMP and ADP that became significant by 20-30 minutes, which then remained relatively constant up to 50 minutes. Thus, AMPK activation and ACC phosphorylation showed a temporal correlation with the increases in AMP and ADP during glucose oxidase treatment. Taken together, these data suggest that AMPK activation by H_2O_2 might be mediated by changes in adenine nucleotide content.

3.2.2 Effect of glucose oxidase in cells expressing an AMP-insensitive mutant of AMPK

In order to confirm that the mechanism of activation of AMPK by H_2O_2 relies on the increase of AMP: ATP and ADP: ATP ratios, we used HEK-293 cells stably expressing a FLAG-tagged WT γ2-AMPK or its RG mutant. We treated these cells with glucose oxidase for increasing times, immunoprecipitated AMPK using anti-FLAG antibody, and measured kinase activity in the precipitates.

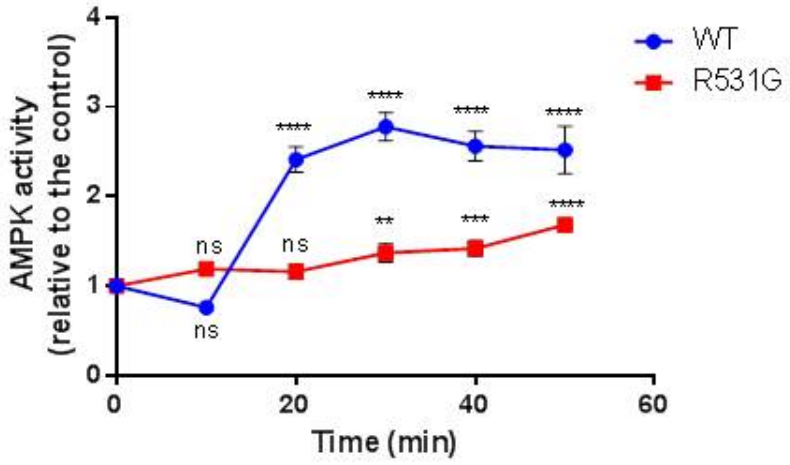

Figure 3.4. Activation of AMPK by glucose oxidase is greatly reduced but not completely lost, in RG cells. WT and RG cells were treated with glucose oxidase. Immunoprecipitation with anti-FLAG antibody was performed and AMPK activity was measured by kinase assays. Data represent 3 biological replicates ±SEM where the normalized value of 1 corresponds to a basal activity of 0.2 nmol/min/mg for both wild type and RG cells. **** = $P \leq 0.0001$.

In WT γ2 cells, treatment with glucose oxidase promoted a large activation of AMPK between 10 and 20 minutes that was sustained for up to 50 minutes (Figure 3.4). The activation of AMPK in RG cells was much less than that in WT cells at all time points, consistent with the idea that AMPK activation by H_2O_2 was mediated primarily by increases in AMP or ADP. However, unexpectedly, when RG cells were treated with glucose oxidase, a small but significant activation of AMPK could still be observed at later time points. Data shown in Figure 3.5 (A, B and C) suggest a possible explanation for the apparent discrepancies between the results previously obtained in our laboratory (Hawley et al, 2010) and our new findings. When WT and RG cells were treated with increasing concentrations of H_2O_2, added to the cell medium as a single bolus, and AMPK activity measured after 1 hour, only WT cells showed a dose dependent increase in AMPK activity, while no response could be detected in RG cells as previously reported (Hawley et al, 2010) (Figure 3.5A). However, when a time course was performed using with 1mM H_2O_2, maximal activation of AMPK in WT cells was detected after 10 minutes after which it declined although a > 2-fold activation was still observed after 60 minutes. A small but significant activation of AMPK was also seen in RG cells after 10 minutes of incubation with H_2O_2, but this had declined back to baseline by 60 minutes (Figures 3.5B and C).

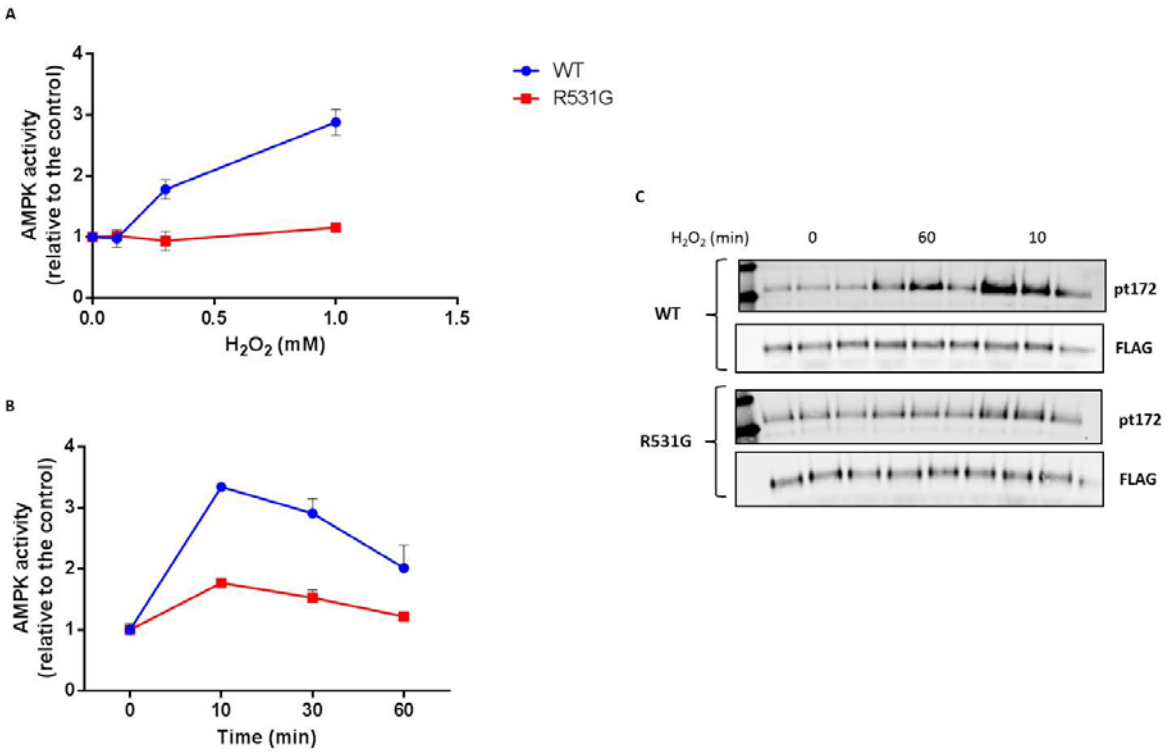

Figure 3.5. Effect of H_2O_2 on WT and RG cells. **(A)** Cells were treated with increasing concentrations of H_2O_2 for 60 minutes and the lysates were analysed by IP-kinase assay. **(B)** Cells were treated with 1 mM H_2O_2 for increasing time and AMPK activity measured. **(C)** Cells were treated with a bolus of H_2O_2 for 0, 10 and 60 minutes. Cell lysates were immunoprecipitated with the anti-FLAG antibody and AMPK phosphorylation evaluated by Western blot analysis with anti-pT172 and anti-FLAG antibodies. Graphs represent the average of 3 independent experiments and the error bars represent the SEM. The normalized value of 1 corresponds to a basal activity of 0.5 nmol/min/mg in the wild type cells and of 0.2 nmol/min/mg in the RG cells.

When we measured the concentration of H_2O_2 present in the cell medium, either after treatment with a single bolus of H_2O_2 or during incubation with glucose oxidase, it was clear that a single dose of H_2O_2 was rapidly metabolized by HEK-293 cells, presumably by cellular catalases and/or peroxidases. Even though the amount of H_2O_2 added had been calculated to yield a final concentration of 1 mM, the maximal concentration detected (after 2 minutes) was only 60 μM, and it then declined extremely rapidly and became completely undetectable by 60 minutes. By contrast, during incubation with glucose oxidase, the concentration of H_2O_2 increased up to 5 minutes and then

reached a constant level of around 10-20 μM (Figure 3.6). This is presumed to represent a steady state where the rate of production of H_2O_2 by glucose oxidase is balanced by its rate of breakdown by cellular catalases and/or peroxidases.

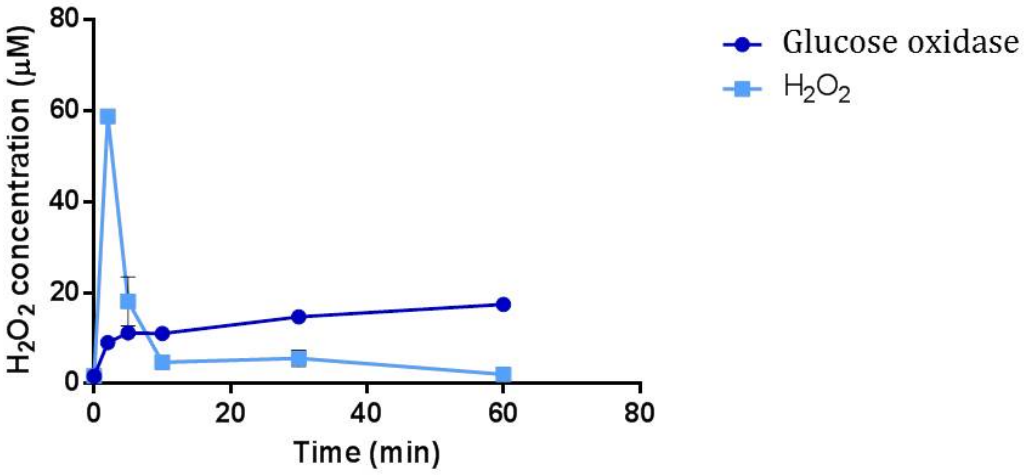

Figure 3.6. H_2O_2 measurements after either H_2O_2 treatment or incubation with glucose oxidase. WT HEK-293 cells were treated with glucose oxidase or with a bolus of H_2O_2 (calculated to yield a final concentration of 1 mM) for increasing time points. H_2O_2 concentrations were measured using the OxiSelectTM Hydrogen Peroxide Assay Kit. Data represent the average of 3 biological replicates ±SEM.

Taken together, these results suggest that Hawley et al (2010) may have missed the small degree of AMPK activation by H_2O_2 in RG cells because they were using a standard incubation time of 60 minutes, by which time cellular antioxidant systems had almost completely metabolized H_2O_2 and had reversed any oxidative damage that might have affected cellular ATP levels. The small but significant amount of AMPK activation we observed in RG cells at later time points suggests that AMPK activation by H_2O_2 might happen part through an AMP/ADP-independent mechanism, perhaps through the direct oxidation of AMPK as suggested by Zmijewski et al (2010). However,

the much larger degree of activation in the WT cells by both H_2O_2 and glucose oxidase treatment (Figures 3.4 and 3.5) suggests that the primary effect of oxidative stress on AMPK is due to changes in adenine nucleotide ratios.

3.2.3 AMPK activation by H_2O_2 is prevented by catalase

To confirm that the effect of glucose oxidase was mediated by H_2O_2, we pre-treated WT and RG cells with catalase, prior to the addition of either glucose oxidase or H_2O_2. Figures 3.7A and 3.7B show that catalase treatment did not have any effect on AMPK on its own. However, it completely prevented the increase in AMPK activity and Thr172 phosphorylation observed in response to either glucose oxidase or H_2O_2, in both WT and RG cells, thus confirming that the glucose oxidase effect on AMPK is mediated by H_2O_2.

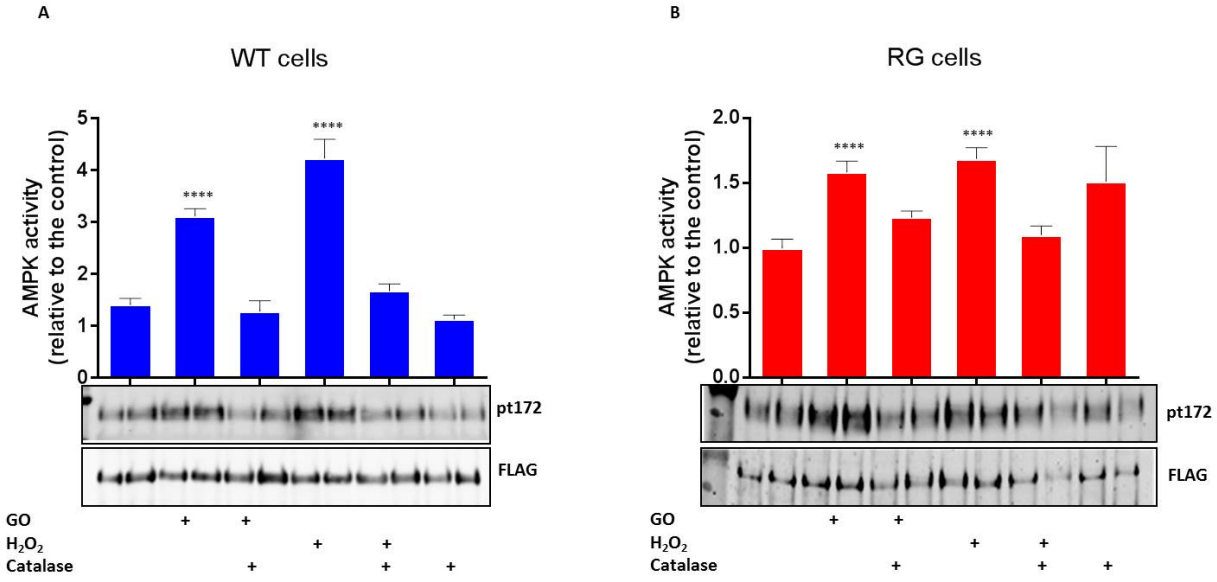

Figure 3.7. Catalase reverts the effect of both H_2O_2 and glucose oxidase. WT **(A)** and RG **(B)** cells were treated with either glucose oxidase or H_2O_2 in the presence or in absence of 5000U/ml of catalase. Lysates were immunoprecipitated with the anti-FLAG antibody and AMPK activity was evaluated either by Western blot analysis or by kinase assay. Each graph represents the average of 3 independent experiments where the error bars represent the SEM. In (A) and (B) control AMPK activity corresponds to 0.4 nmol/min/mg and 0.1 nmol/min/mg respectively. **** = $P \leq 0.0001$.

Given that activation of AMPK by H_2O_2 can be prevented by catalase, we wondered whether the effect of H_2O_2 on cellular nucleotides was a reversible process. We incubated WT cells with H_2O_2, extracted nucleotides and measured the ADP: ATP ratio at various time points. As shown in Figure 3.8, we observed a large increase in ADP: ATP ratio at early time points, but this had reverted to the baseline values by 90 minutes.

This result further confirms that the concentration of H_2O_2 used in our experiments only temporarily affects the cellular content of adenine nucleotides, and hence AMPK activity.

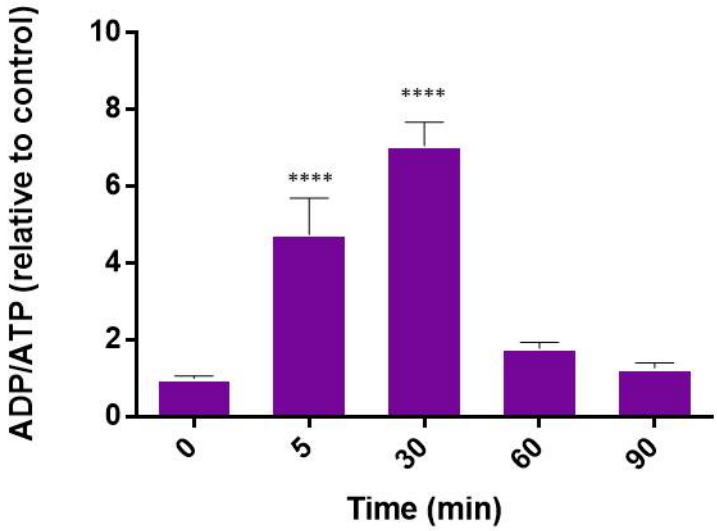

Figure 3.8. The increased ADP:ATP ratio induced by H_2O_2 is transient. A time course was performed with HEK-293 cells treated with H_2O_2 for 5, 30, 60 and 90 minutes. Nucleotides were extracted and ADP/ATP ratio was measured. Data represent the average of 3 biological replicates ± SEM. The normalized value of 1 corresponds to 0.3. **** = $P \leq 0.0001$.

3.2.4 Pre-treatment with STO609 affects AMPK activation by glucose oxidase in HeLa and G361 cells but not in HEK-293 cells

Although the results in the previous section suggest that H_2O_2 activates AMPK primarily by increasing AMP:ATP and ADP:ATP ratios, there must be a secondary nucleotide-independent mechanism of activation that explains the small activation observed in the RG cells either during prolonged treatment with glucose oxidase or during short-term treatment with H_2O_2. We decided to investigate whether CaMKKβ is involved in this second pathway. To this aim we pre-treated WT and RG cells with the CaMKKβ inhibitor STO609, and then incubated with glucose oxidase or the Ca^{2+} ionophore A23187.

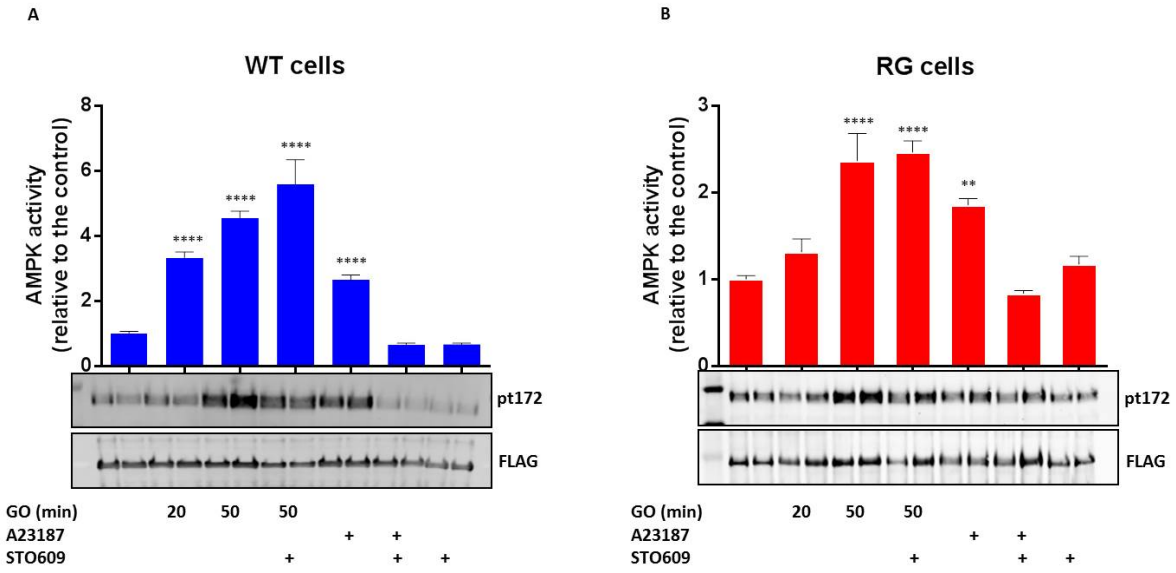

Figure 3.9. AMPK activation by H_2O_2 is not CaMKKβ-dependent. WT **(A)** or RG **(B)** HEK-293 cells were treated with glucose oxidase, STO609 or A23187. Anti-FLAG immunoprecipitation was performed and AMPK activation was evaluated through kinase assay and Western blot analysis. Treatment with A23187 was used as positive control, while treatment with 25 μM STO609 alone was used as negative control. Cells pre-treated with STO609 and then treated with A23187 were used as control for the efficacy of the inhibitor STO609. 3 independent experiments were performed to generate each graph. Error bars represent the SEM. The normalized value of 1 represents a basal AMPK activity of 0.3 nmol/min/mg in wild type cells and of 0.4 nmol/min/mg in RG cells. ** = P ≤ 0.01; **** = P ≤ 0.0001.

Figure 3.9 (A and B) shows that STO609 is able to inhibit activation of AMPK by the Ca^{2+} ionophore A23187, as expected. However no significant loss of activity could be detected in response to glucose oxidase after pre-treatment with the inhibitor, either in WT or in RG cells, suggesting that increased AMPK activity and Thr172 phosphorylation in response to oxidative stress was not dependent on CaMKKβ under these conditions. However, when we pre-treated either HeLa or G361 cells, which are LKB1-null human cell lines (derived originally from a cervical cancer or a melanoma respectively), with STO609, glucose oxidase-induced AMPK activation was greatly reduced. These findings (Figures 3.10 A and B) indicate that the mechanism of AMPK activation by H_2O_2 in these LKB1-null cell lines does require CaMKKβ as an alternative upstream kinase.

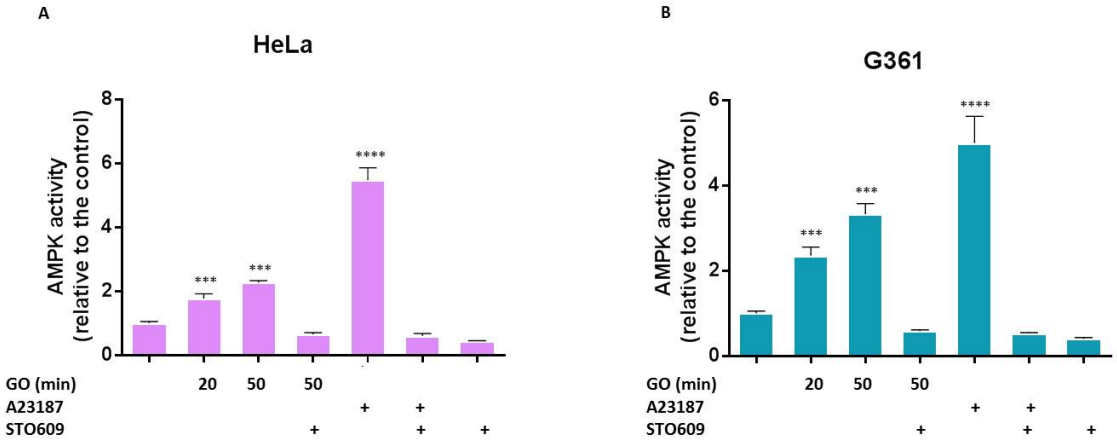

Figure 3.10. CaMKKβ mediates AMPK activation by H_2O_2. HeLa **(A)** and G361 **(B)** were incubated with glucose oxidase, STO609 and A23187. IP-kinases assay using anti-α1/α2 antibodies were performedon cell lysates. Data represent 3 biological replicates ± SEM. In (A) and (B) control AMPK activities correspond to 0.02 nmol/min/mg and 0.03 nmol/min/mg respectively. ** *= P ≤ 0.001; **** = P ≤ 0.0001.

3.2.5 Hydrogen peroxide treatment inhibits Thr172 dephosphorylation in intact cells

The results in the previous section suggested that treatment of cells with glucose oxidase to generate oxidative stress could cause activation of AMPK irrespective of which upstream kinase (LKB1 or CaMKKβ) was being utilized. This suggested that oxidative stress might be acting by affecting the rate of Thr172 dephosphorylation, rather than phosphorylation. To examine this, we used HeLa cells, which lack LKB1 and thus are the ideal model system to study this hypothesis. In these cells, basal phosphorylation of Thr172 is very low, but can be increased by activation of CaMKKβ, following the addition of the Ca^{2+} ionophore A23187. After 30 minutes of A23187 treatment, Thr172 phosphorylation then reaches a new, higher steady state level. The addition of STO609 will then instantly inhibit CaMKKβ, and, since the cells lack LKB1 or any other upstream kinase, any change in AMPK activity and Thr172 phosphorylation subsequently observed must be due to dephosphorylation rather than promotion of phosphorylation. This experimental procedure, which is summarized in Figure 3.11, gave us a unique opportunity to measure the rate of Thr172 dephosphorylation in intact cells.

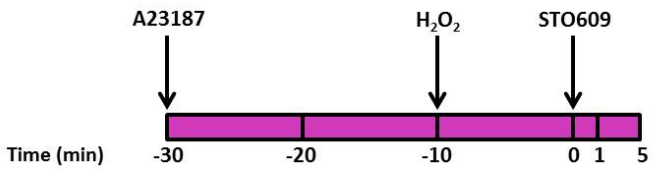

Figure 3.11. Protocol for assays to monitor Thr172 dephosphorylation in intact cells

By 1 minute after the addition of STO609 to the cell medium, an almost complete dephosphorylation of Thr172, and inactivation of AMPK had occurred. The speed with which this occurred indicates that the phosphate on Thr172 is turning over very rapidly, even under conditions where upstream kinase(s) are active. After 10 minute treatment with H_2O_2 (following the pre-treatment with A23187), there was a significantly increased steady state phosphorylation of Thr172 and AMPK activation compared with pre-treatment with A23187 alone. This could either have been due to activation of CaMKKβ or inhibition of protein phosphatase(s) activing on Thr172.

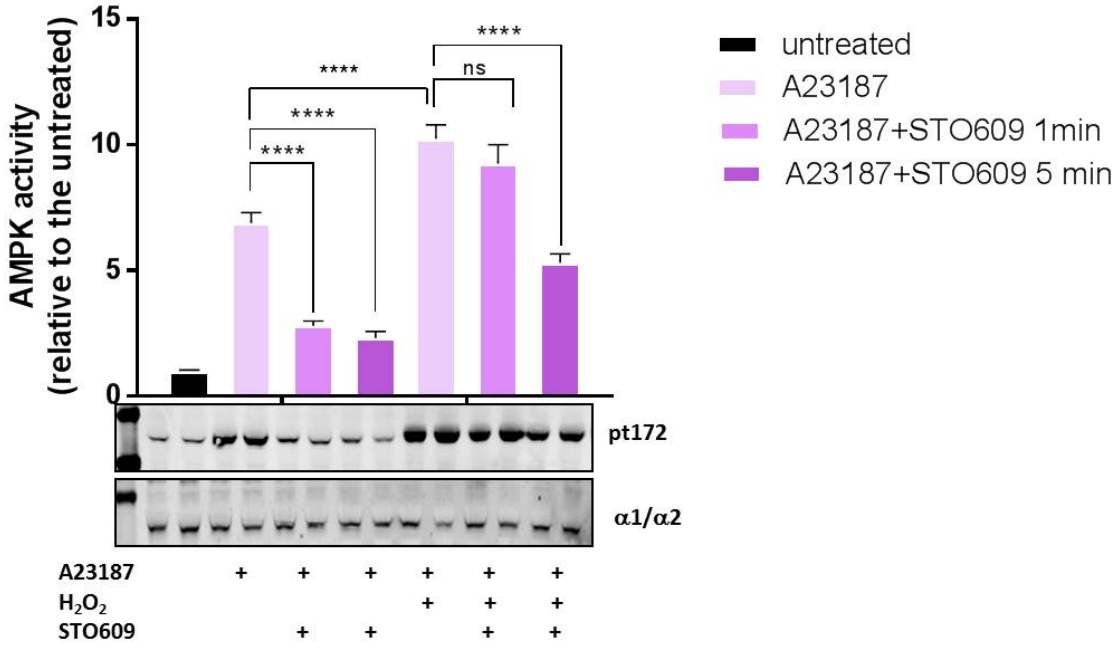

Figure 3.12. H_2O_2 promotes Thr172 phosphorylation by inhibiting dephosphorylation. HeLa cells were treated with 10 μM A23187 for 20 minutes. H_2O_2 (1 mM) was then added for 10 minutes. Cells were then treated with 25 μM STO609 for 1 and 5 minutes and AMPK phosphorylation was evaluated by Western blot analysis using anti-pT172 and anti-α1/α2 antibodies. Lysates were immunoprecipitated with anti-α1/α2 antibody and AMPK activity measured by kinase assay. The graph represents the average of 3 independent experiments with the error bars representing the SEM. Control AMPK activity corresponds to 0.007 nmol/min/mg. **** = P ≤ 0.0001.

However, H_2O_2 treatment also very significantly reduced the rate of dephosphorylation observed following the addition of STO609, when CaMKKβ should be completely inactive (Figure 3.12).Compared with the controls not treated with H_2O_2, there was a modest and insignificant Thr172 dephosphorylation and inactivation 1 minute after addition of STO609, although the effects were more substantial after 5 minutes. There results suggest that H_2O_2 was mediating its effects, at least in part, by the inhibition of Thr172 dephosphorylation.

We also tested the effect of H_2O_2 and A23187 on cellular nucleotide content. As expected, treatment with H_2O_2 causes a large increase in ADP: ATP ratio in HeLa cells.

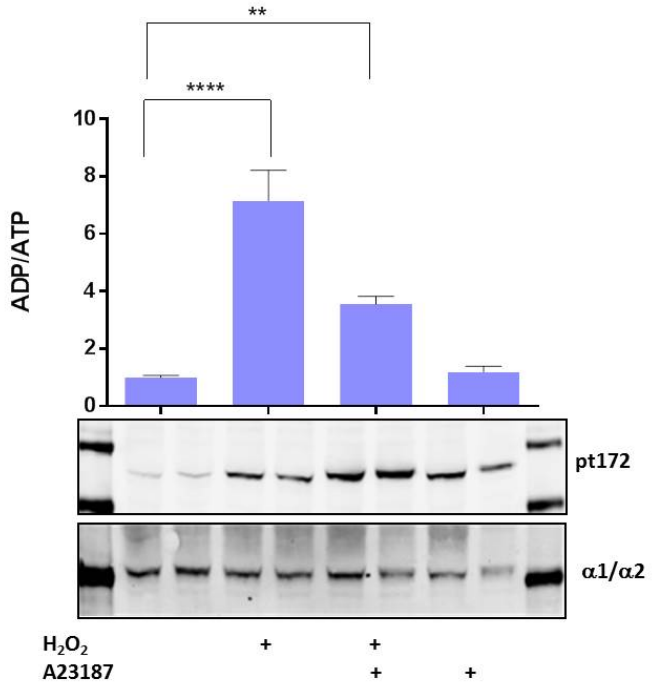

Figure 3.13. Effect of H_2O_2 and A23187 on ADP/ATP ratio in HeLa cells. HeLa cells were stimulated with H_2O_2, A23187 or both. Nucleotides were extracted and ADP/ATP ratio evaluated via capillary electrophoresis. Thr172 phosphorylation and total AMPK were detected by Western blot analysis using anti-pT172 and anti-α1/α2 antibodies. Data represent the average of 3 biological replicates ± SEM. ** = P ≤ 0.01; **** = P ≤ 0.0001.

Surprisingly, treatment with A23187 reduced the effect of subsequent H_2O_2 addition, despite having no effect on its own. The combination of A23187 plus H_2O_2 also

resulted in higher Thr172 phosphorylation compared with treatment with either agent alone (Figure 3.13).

3.3 Discussion

The findings shown in this chapter clarify some of the apparent discrepancies between the previous study from our laboratory (Hawley et al., 2010) and the work of Zmijewski et al (2010). Hawley et al (2010) reported that a single dose of H_2O_2 activated wild type AMPK while having no effect on the R531G mutant, suggesting that the mechanism of activation was entirely explained by changes in cellular nucleotides. However, at that time it was not realized how rapidly H_2O_2 is metabolized by cultured cells (Figure 3.6) and a standard incubation time of 60 minutes was used, as used for all of the other activating agents under study. Here we show that a small but significant activation of the R531G mutant-AMPK can be detected after 10 minutes of treatment with H_2O_2, but the effect is reversed by 60 minutes (Figures 3.5B and C). However, Figure 3.6 shows that a single dose of H_2O_2 is metabolized very rapidly by HEK-293 cells (presumably by cellular catalase and/or peroxidases), and that H_2O_2 was almost completely eliminated from the medium after only 10 minutes. The findings that both ADP:ATP ratio (Figure 3.8) and AMPK activity in the RG cells (Figure 3.5 B) had returned to baseline by 60 minutes suggest that the cells must also be able to rapidly reverse any oxidative damage to cellular ATP-producing pathways. In the wild type HEK-293 cells, AMPK activation was also significantly higher 10 minutes after H_2O_2 addition compared with that observed after 60 minutes (Figure 3.5 B). In their work Zmijewski et al (2010) used a different method to stimulate cells with H_2O_2, incubating cells with glucose oxidase to generate H_2O_2 from glucose present in the cell medium. Figure 3.6

shows that addition of glucose oxidase rapidly establishes a constant concentration of 10-20 μM H_2O_2 in the medium, presumably because the rate of H_2O_2 production by glucose oxidase is balanced by its destruction by cellular catalase and/or peroxidases. This is likely to be a better model for physiological oxidative stress than addition of a single bolus of H_2O_2 to the medium, which we showed to cause an initially much higher concentration of H_2O_2 in the medium (although much lower than expected based on the dilution involved) but which was extremely short-lived, almost all of it having disappeared by 10 minutes (Figure 3.6). Using this approach we could observe AMPK activation after 20 minutes of incubation with glucose oxidase (Figures 3.2A and B), similar to that observed by shown by Zmijewski et al (2010). Moreover, using an incubation time of 50 minutes a small but still significant effect was also observed in RG cells (Figure 3.4). It is possible that this small degree of activation of the RG mutant, which we observed with both glucose oxidase and H_2O_2 treatment although with different time courses, could be caused by a direct oxidation of cysteine residues on AMPK, as suggested by Zmijewski et al (2010). However, from our data it is clear that the much larger increase in AMPK activity that we observed in WT cells, correlated with large increases in ADP:ATP and AMP:ATP levels (Figure 3.3), strongly suggesting that the primary activation mechanism depends on changes in cellular nucleotides. Zmijewski et al (2010) claimed that there was no increase in ADP:ATP ratio after incubation of HEK-293 cells with glucose oxidase for 40 minutes, in direct contrast to our results (Figure3.3). The reason for this discrepancy remains unclear. A direct comparison between their nucleotide measurements and ours is difficult partly because two different methods for extracting cellular nucleotides were described by Zmijewsky et al., but it is unclear which of the two were used in the results presented. Furthermore, they did not report absolute ADP:ATP ratios, but only presented their

results as relative to the control. It has been suggested that concentrations of H_2O_2 causing a mild oxidative stress (between 0.7 to 20 μM) might induce a temporary growth arrest in cultured mammalian fibroblasts (Davies, 1999). This mechanism could be considered as a defence against oxidative stress; during H_2O_2-induced growth arrest, progress through the cell cycle is inhibited, protecting the DNA from potential oxidative damage while it is particularly vulnerable during replication in S-phase. The expression of many housekeeping genes is also halted, while the expression of a select group of stress-response genes is induced. AMPK activation by oxidative stress could be included in such a context as a mechanism through which cells try to re-establish their energetic balance, after disturbance by oxidative stress. However, it is worth considering that, once activated, AMPK causes arrest in G1 phase before DNA replication (Imamura et al, 2001). It is possible that AMPK itself mediates the temporary growth arrest induced by H_2O_2. Figure 3.8 clearly shows that after 60 minutes of incubation with H_2O_2, ADP:ATP ratios had returned to the baseline, perhaps due to the activated-AMPK promoting alternative pathways to produce ATP and switching off pathways consuming ATP. Interestingly, the effect of H_2O_2 on AMPK appeared to be independent of the upstream kinase being utilized. In HEK-293 cells LKB1 appeared to be the kinase primarily phosphorylating Thr172, since pre-treatment with STO609, a CaMKKβ inhibitor, did not affect either activation or phosphorylation of AMPK induced by glucose oxidase (Figures 3.9 A and B). This predominant role of LKB1 may be because it appears to be constitutively active, whereas CaMKKβ is only active under conditions where intracellular Ca^{2+} has increased. Despite this, in cells lacking LKB1 such as HeLa or G361 cells, glucose oxidase-induced AMPK activation was prevented by STO609, suggesting that, in the absence of LKB1, CaMKKβ is required for the mechanism of activation of AMPK by oxidative stress. This is consistent with the

idea that the major effect of increased cellular AMP:ATP or ADP:ATP ratio on net Thr172 phosphorylation is due to inhibition of Thr172 dephosphorylation, which occurs irrespective of the upstream kinase that is being used for Thr172 phosphorylation. It was previously proposed that AMPK activation by H_2O_2 in HeLa cells is mediated by CaMKKβ (Woods et al, 2005). Moreover, it has been suggested that CaMKII, a member of the closely related family of Ca^{2+}/Calmodulin-dependent kinases (CaMKs), is activated by H_2O_2 by the oxidation of two methionine residues in its regulatory subunit (Burgoyne et al, 2013). This leaves open the possibility of a direct effect of H_2O_2 on CaMKKβ.

There might be several different explanations for the H_2O_2-induced increase in AMP and ADP levels observed here. One possibility is that oxidative stress induces DNA damage, resulting in poly ADP-ribose polymerase (PARP) activation and the consequent depletion of cellular NAD^+, leading to inhibition of glycolysis (Kim et al, 2005). Glycolysis might also be inhibited by promotion of ADP-ribosylation of glyceraldehyde-3 phosphate dehydrogenase by oxidative stress (Dimmeler et al, 1992). However, by measuring cellular oxygen uptake, our laboratory previously provided evidence that H_2O_2 impairs the function of the mitochondrial respiratory chain (Hawley et al.,2010), which could also be the cause of increases in AMP and ADP. AMP and ADP bind to the AMPK-γ subunits and increase AMPK phosphorylation in part by protecting Thr172 from dephosphorylation. This study provides direct evidence that H_2O_2 inhibits AMPK dephosphorylation in intact cells. In HeLa cells the Ca^{2+} ionophore A23187 stimulates AMPK activation entirely through CaMKKβ. Subsequent addition of the CaMKKβ inhibitor STO609 caused rapid Thr172 dephosphorylation that was almost complete within 1 minute (indicating that the phosphate on Thr172 is turning over

very rapidly, even when phosphorylation levels are high). However, when cells were treated with H_2O_2 for 10 minutes prior to treatment with STO609, an initial increase in steady state AMPK activation and phosphorylation was observed, as well as a large reduction in the rate of inactivation and dephosphorylation on subsequent addition of STO609 (Figure 3.12). We propose that this effect is due to the inhibition of dephosphorylation caused by the binding of AMP or ADP to AMPK, although we cannot rule out the possibility that H_2O_2 might also have a direct inhibitory effect on the protein phosphatases that dephosphorylate Thr172. There are few reports of regulation of Ser/Thr phosphatases by oxidative stress compared to the well-known redox sensitivity of protein tyrosine phosphatases, which has been reported to have a clear biological importance in different experimental models (Salsman et al, 2005). However, some reports suggest that H_2O_2 might have an inhibitory effect also on Ser/Thr phosphatases (O'Loghlen et al, 2003; Rao & Clayton, 2002).

Interestingly, the increase in ADP:ATP ratio observed in HeLa cells following H_2O_2 treatment appeared to be less pronounced when the cells had been pre-treated with A23187 (Figure 3.13). This suggests that AMPK activation by A23187 had already induced metabolic changes, such as acute activation of glucose uptake and glycolysis (Hardie et al, 2012b) that helped the cells to better deal with the oxidative damage caused by H_2O_2.

While this work was in progress, it was shown that oxidative stress can inhibit (rather than activate) AMPK in primary cardiac myocytes (Shao et al, 2014). AMPK α-subunits were identified as targets of thioredoxin-1 (Trx1), and when Trx1 was knocked out in mice, the normal AMPK activation that occurred during cardiac ischemia was prevented. Two cysteine residues were identified (Cys130 and Cys174) in the α subunit

whose reduction by Trx1 protected AMPK from inhibition during glucose deprivation or cardiac ischemia. These cysteines differ from those suggested to be involved in AMPK activation during oxidative stress by Zmijewski et al (2010). Moreover, Shao et al (2014) could not find any evidence for oxidative modification of AMPK when HEK-293 cells were treated with H_2O_2, despite an increase in Thr172 phosphorylation. This is consistent with our conclusions that the primary effect of oxidative stress on AMPK in HEK-293 cells was indirect, due to changes in cellular adenine nucleotides rather than direct oxidative modification of AMPK. Shao et al (2014) speculated that immortalized cell lines, such as the HEK-293, HeLa and G361 cells used in our studies and those of Zmijewski et al (2010) might have up-regulated anti-oxidant defences compared to primary cells, and this might prevent the inactivation of AMPK during oxidative stress that they observed in primary cardiac myocytes.

Chapter 4: A novel regulatory site on AMPK

4.1 Introduction

The overall structure of AMPK, along with the description of its subunits, was extensively discussed in Chapter 1 of this thesis. However, while the work for the completion of this PhD was being carried out, a paper describing a new crystal structure of an almost complete human $\alpha 2\beta 1\gamma 1$ heterotrimer was published (Xiao et al, 2013). The work of Xiao and colleagues provided new insights in the understanding of the structure of AMPK, together with the discovery of a new ligand binding pocket located at the interface between the α and the β subunits. This study represented a real breakthrough in the field, allowing a better comprehension not only of the structure of AMPK itself, but also of its different regulatory mechanisms. According to these latest discoveries, the structure of AMPK can be divided into two major modules: the "catalytic module", containing the α kinase domain and the β-CBM, and the "nucleotide-binding module" formed by the γ subunit and the α and β C-terminal domains. These two modules are connected by: (i) the α-AID and the α-linker, the latter being a region of extended polypeptide that connects the α-AID to the α-CTD; and (ii) the linker between the β-CBM and the β-CTD, which is poorly resolved in all existing structures. Thr172 is located in the cleft between these two modules, in a position where access to upstream kinases and phosphatases might be particularly sensitive to conformational changes. Figure 4.1 provides a schematic representation of the up to date structure of AMPK.

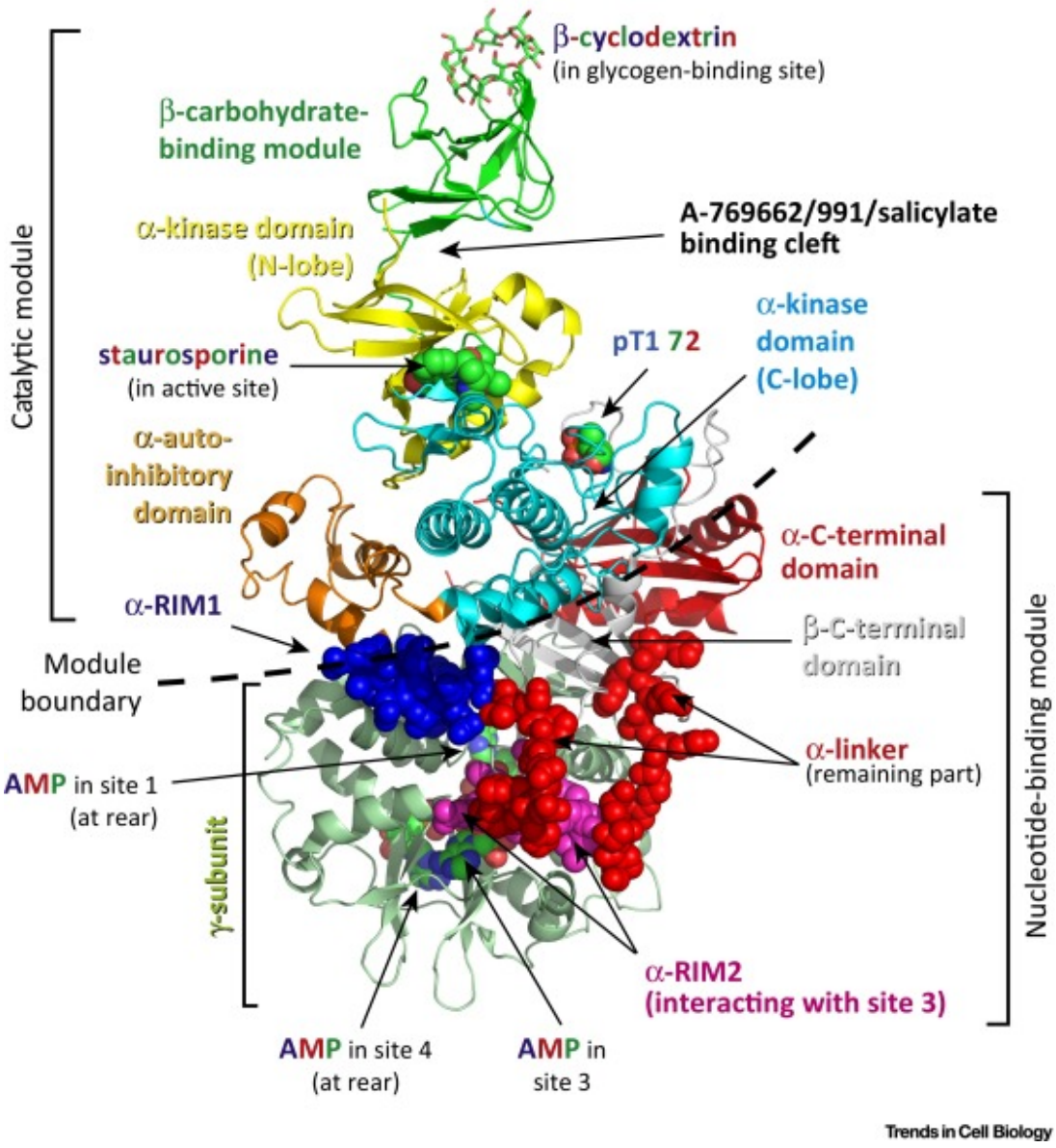

Figure 4.1 Crystal structure of the human α1β2γ1 AMPK. This figure was obtained from Hardie *et al.* (2015). It shows how AMPK complex can be divided into two main modules: a catalytic module (top part of the structure), containing the kinase domain and part of the CBM of the β subunit, and a nucleotide-binding module (bottom part of the structure), comprising the γ subunit and the C-termini of both α and β subunits.

The recent crystal structure of human AMPK, along with subsequent studies, provided new interesting details of the structure of the α, β and γ subunits, which will be now discussed briefly.

4.1.1 New insights into AMPK structure and regulation

Within the α subunit of AMPK, the kinase domain at the N terminus contains the small N-lobe and larger C-lobe of a typical serine/threonine kinase domain. As previously discussed in Chapter 1, the kinase domain of the α subunit is followed by the auto-inhibitory domain (α-AID) (Pang et al, 2007). Structures of isolated rat and human AIDs showed that they form a compact bundle of three α helices (Chen et al, 2013). Studies performed on a construct from the *Schizosaccharomyces pombe* orthologue showed that when AMPK is in an inactive conformation, helix α3 of the α-AID interacts with the kinase domain on the opposite surface to the active site, forming contacts with residues in the C-helix of the N-lobe and the E-helix of the C-lobe. This seems to maintain the kinase domain in an inactive conformation (Chen et al, 2009). Although in the structure presented by Xiao et al (2013) the α-AID was not fully resolved, comparison with the *S.pombe* α-AID structure (Chen et al, 2009) made it clear that the α-AID had undergone a large rotation so that helix α3 interacted with the γ subunit, rather than with the N- and C-lobes of the kinase domain. This was further confirmed by Li et al (2015) by structures of a kinase domain-AID construct from human α1, and of a complete human α1β2γ1 heterotrimer. The rotation seems to be caused by the binding between the AMP-bound γ subunit and the α-linker, which together with the α-AID forms a hinge between the catalytic module and the nucleotide-binding module.

One of the most interesting features revealed by the new heterotrimer structure is that the β-CBM appears to be located next to the N-lobe of the kinase domain, forming a cleft between the two subunits. One of the five β-sheets forming the core of the N-terminal domain of the kinase domain packs against a pair of antiparallel β-strands of the β-CBM (Xiao et al, 2013) and this cleft seems to be the binding site for A769662

and 991 (Xiao et al, 2013). Despite not being directly involved in the binding of those ligands, Ser108 within the β-CBM, once phosphorylated, appears to stabilize this binding cleft by forming interactions between the CBM and the N-lobe of the kinase domain. In the new crystal structure, an α-helix immediately C-terminal to the β-CBM was also identified for the first time. This α-helix interacts with the C-helix of the N-lobe of the kinase domain by forming hydrophobic contacts, and it seems that this interaction may transmit the effects of binding of A769662 and 991 to the active site. In fact, one of the crucial steps for the activation of many kinases is a change in the position of the N-terminal end of the C-helix (Taylor & Kornev, 2011).

Already in 2011 Xiao and colleagues had suggested that the α-linker wrapped around the face of the γ subunit containing sites 2 and 3 (Xiao et al, 2011). However, it was pointed out that their mapping of the sequence of the α-linker to the electron density may have been incorrect (Chen et al, 2013). A re-interpretation of the data (Xin et al, 2013) suggested that a well-conserved sequence motif of the α-linker (α-regulatory subunit interacting motif-1, α-RIM1) made contact with site 2, while another conserved segment, α-RIM2, contacte site 3 with its bound AMP (this re-interpretation was later accepted by Xiao et al (2013)). It has been proposed that the binding of α-RIM1 and α-RIM2 to the γ subunit when AMP is bound in site 3, would force the α-AID to dissociate from the kinase domain, thus preventing its inhibitory effects. This change may also pull the catalytic and the nucleotide-binding modules together, so that protein phosphatases could no longer easily access Thr172, thus protecting AMPK from dephosphorylation and inactivation.

The new features discussed so far along with additional data shown by Li et al (2015) also provide a new model for AMPK regulation by adenine nucleotides. This presents

AMPK as a molecular machine with four interconnected moveable parts: the N- and C-lobes of the kinase domain, the β-CBM, the α-AID and αRIM. In the presence of ATP, the interaction of the α-AID and α-linker with the nucleotide-binding module of AMPK is weak, and the α-AID is free to engage its inhibitory interaction with the kinase domain. This locks the kinase domain in an open position with a low catalytic activity. Furthermore, the release of the α-linker from the nucleotide-binding module makes AMPK less compact and more accessible to protein phosphatases. On the other hand, in the presence of AMP, both α-AID and α-linker interact with the nucleotide-binding module, thus releasing the kinase domain from inhibition. The N- and C-lobes of the kinase domain are now free to move toward each other to adopt a closed and active conformation (Hawley et al, 2014). The interaction between the α-linker and the γ subunit also means that the catalytic and nucleotide-binding modules are pulled together, making Thr172 less accessible to protein phosphatases. In support of this, luminescence energy transfer measurements with probes attached to the N-termini of the α and γ subunits suggest that the heterotrimer adopts a more compact conformation in the presence of AMP, and a less compact conformation in the presence of ATP (Li et al, 2015).

As previously mentioned, the α2β1γ1 crystal structure presented by Xiao et al (2013) also allowed the identification of the binding site for both A769662 and 991. The same study also provided evidence that two residues in the binding cleft, Lys29 and Lys31 of the α2 subunit, play a fundamental role in the binding of those two activators. Mutation of both Lys29 and Lys31 to alanine resulted in a reduction of more than 25-fold in affinity for 991 and completely abolished binding of A769662 (Xiao et al, 2013).

The aim of this chapter will be to further characterize the newly discovered binding pocket, by mutating Lys29 and Lys31 of the α2 subunit (equivalent to Lys40 and Lys42 in the α1 subunit), in order to confirm that it is the binding site of A769662 and 991 and to test whether it could be the binding site for other known activators of AMPK.

4.2 Results

4.2.1 Purification and phosphorylation of bacterial AMPK

In order to verify whether the newly discovered binding site at the interface between the N-lobe of the α subunit and the β-CBM is where some direct activators of AMPK exert their effects, we started by using a bacterially expressed α1β1γ1 complex. Two lysine residues in the α2 subunit, Lys29 and Lys31, were identified from the crystal structure as critical for the binding of both A769662 and 991. Those two residues correspond to Lys40 and Lys42 in the human α1 subunit. Therefore we produced bacterially expressed complexes carrying single and double mutations of those two lysines. We used site-directed mutagenesis to mutate Lys40 and Lys42 into alanine residues. The wild type and mutant heterotrimers generated also contained a His-tag at the N-terminus of their α subunits, which allowed us to purify the complexes easily. Figure 4.2 A shows the absorbance at 280 nm during the purification of the wild type complex on Ni^{2+}-Sepharose (the procedure was also performed with the mutated heterotrimers).

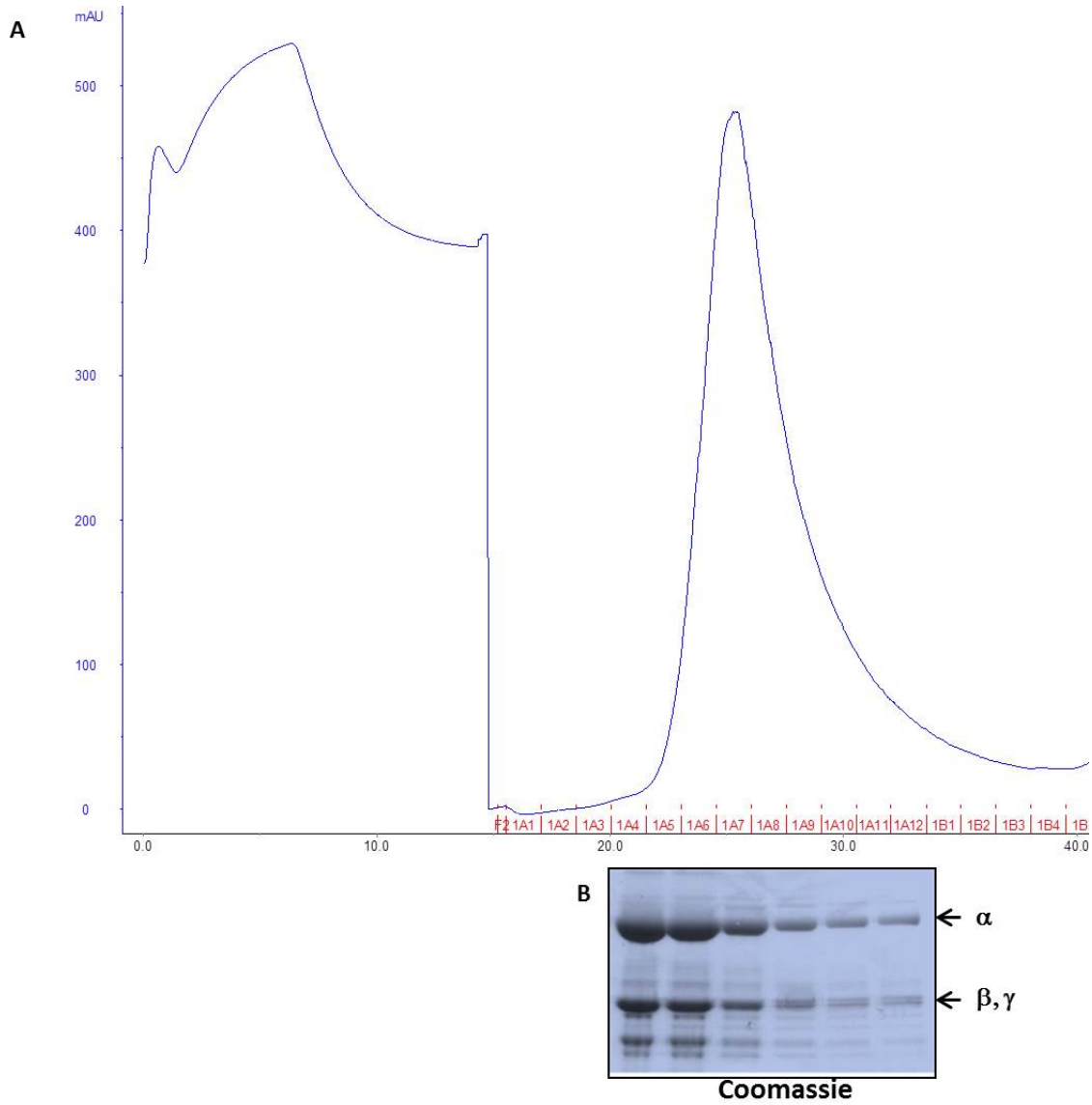

Figure 4.2 Purification of α1β1γ1 heterotrimer. (A) His-tagged α1β1γ1 was expressed in *BL21 E. coli*. Bacteria were lysed in an imidazole-containing buffer. The lysate was ultracentrifuged and applied to a HisTrap HP column, prepacked with Ni^{2+}-containing Sepharose. Elution was performed using a gradient of imidazole. **(B)** Protein elution was detected by absorbance at 280 nm.Relevant fractions were visualized by SDS-PAGE followed by Coomassie Blue staining.

Some of the fractions eluting in the protein, were then loaded on a gel, confirming the presence of AMPK α, β and γ subunits in the fractions analysed (Figure 4.2 B). Bacterially expressed proteins are not phosphorylated on Thr172, and consequently have an extremely low basal activity, so it was necessary to incubate them with an upstream kinase (CaMKKβ) to phosphorylate Thr172 phosphorylation and needed for

AMPK activation. Firstly, the heterotrimeric complexes were incubated with different concentrations of CaMKKβ and MgATP for 30 minutes, to determine by kinase assay the amount of CaMKKβ to give maximal activation of the AMPK complex (Figure 4.3 A).

A large scale reaction was then performed using AMPK and the appropriate quantity of CaMKKβ, ATP and $MgCl_2$. To remove the CaMKKβ, which was GST-tagged, a gel filtration column in line with a glutathione-Sepharose column was used. As can be seen in Figures 4.3 B, all the fractions eluted during the gel filtration step contain AMPK α, β and γ subunits and were free of CaMKKβ, showing that CaMKKβ had been removed by the glutathione-Sepharose column used. At this point we wished to verify that we had equal recoveries of each complex and that the degree of phosphorylation was the same.

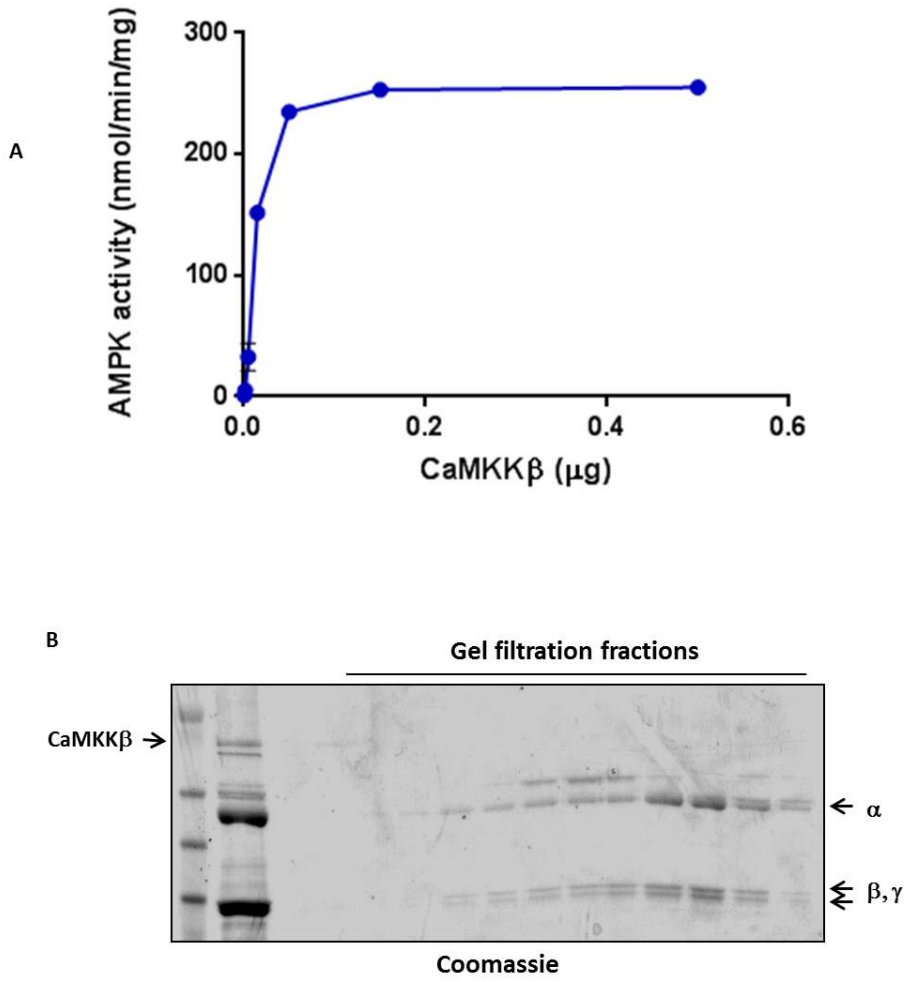

Figure 4.3 Phosphorylation of α1β1γ1 by CaMKKβ. (A) 0.5 μg of α1β1γ1 was incubated with serial dilutions of CaMKKβ. The reaction occurred in the presence of 200 μM ATP and 5 mM $MgCl_2$ for 30 min. Aliquots of each reaction was assayed for AMPK activity using the SAMS peptide. **(B)** α1β1γ1 (6 mg) was incubated with GST-CaMKKβ (0.6 mg), 200 μM ATP and 5 mM $MgCl_2$ in a final volume of 1.8 ml for 30 minutes. The reaction was then applied to a HiLoad 16/60 Superdex 200 column at the end of which a GST-conjugated column was mounted. Selected fractions were subject to SDS-PAGE followed by a Coomassie Blue-staining. As a control, the first lane was loaded with a sample of the reaction before the gel filtration step.

We therefore analysed them by SDS-PAGE followed either by Coomassie Blue staining (Figure 4.4 A) or Western blot analysis (Figure 4.4 B). As shown in the figures, all of the preparations contained almost the same amount of α, β, and γ subunits (note that β1 and γ1 almost co-migrate on SDS-PAGE). Furthermore, phosphorylation of Thr172 was

comparable in the 4 different complexes (Figure 4.4 B top panel). Finally we also performed a kinase assay testing the basal activity of the four bacterial complexes which, as shown in Figure 4.4 C, was comparable for the wild type and the single mutants, while the double mutant seemed to be somewhat less active.

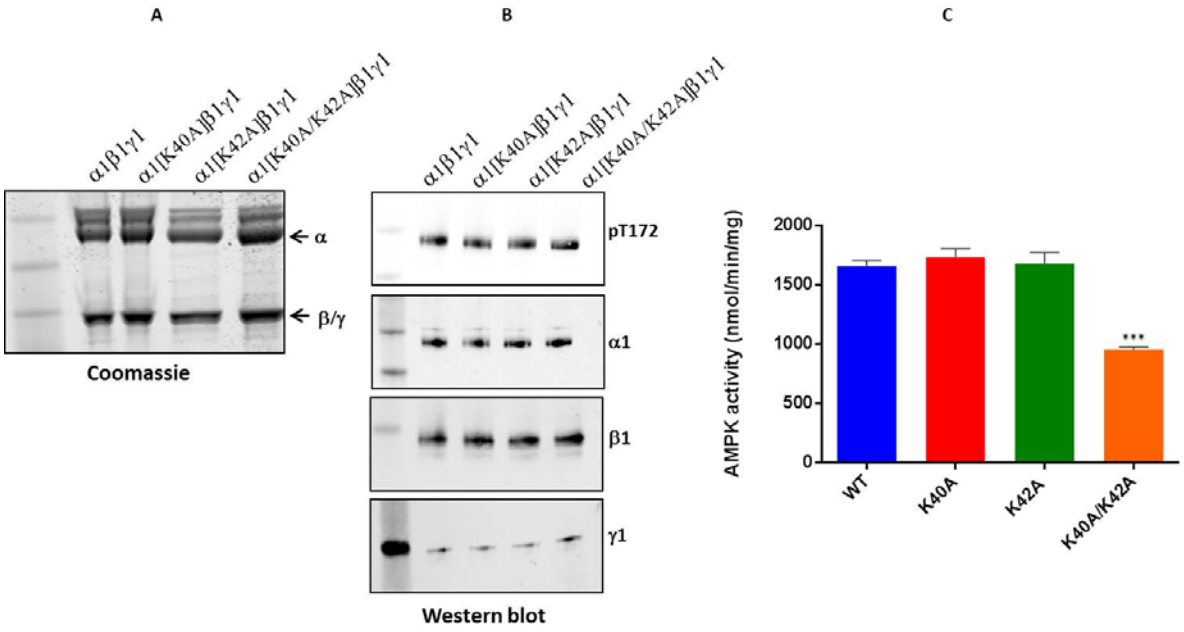

Figure 4.4 Normalization of wild type and mutated α1β1γ1 complex. SDS-PAGE was performed using purified wild type and mutated heterotrimeric α1β1γ1 complexes. **(A)** Coomassie Blue staining. **(B)** Western blot analysis using pT172, α1, β1 and γ1 antibodies. **(C)** The same quantity of wild type and mutated purified proteins was assayed using a standard kinase assay with SAMS peptide. The graph is representative of 3 biological replicates where error bars represent SEM. *** = P≤0.001.

4.2.2 Mutation of Lys40 and Lys42 inhibits activation of bacterial AMPK by A769662

In order to confirm the findings of Xiao et al (2013), we tested the effects of increasing concentration of A769662 with each bacterial complex generated. The wild type protein and the two single mutants respond to A769662 in almost the same manner

showing a maximal degree of allosteric activation of more than 2-fold with an EC_{50} comprised between 0.1 and 0.2 μM, while no response could be detected using the double mutant, confirming that Lys40 and Lys42 are indeed crucial residues for the binding of A769662 and their mutation prevents AMPK allosteric stimulation by A769662. The results are shown in Figure 4.5.

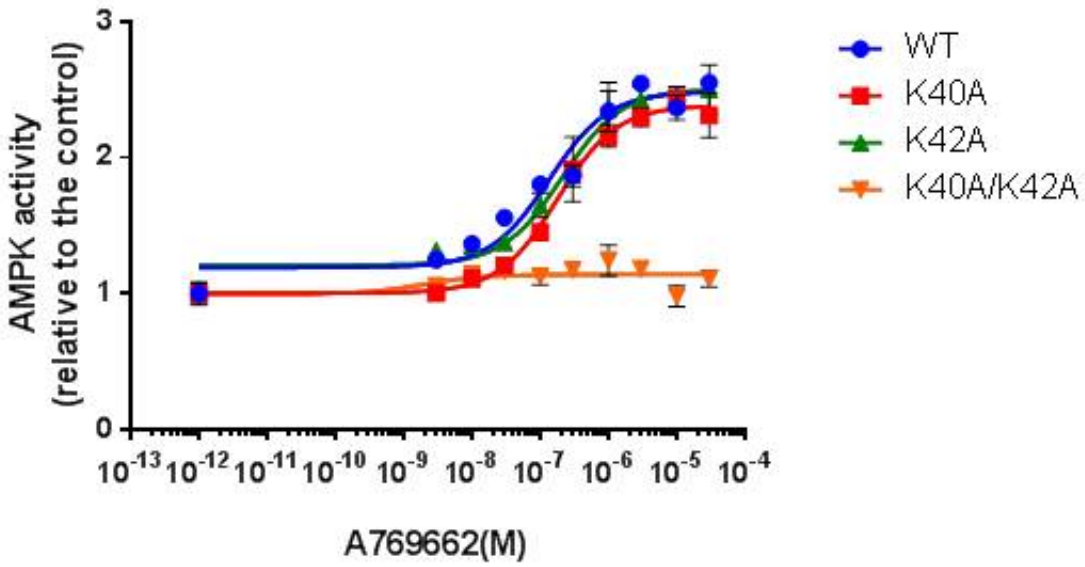

Figure 4.5 A769662 dose response curve with wild type and mutated α1β1γ1 complex. Equal amounts of purified heterotrimeric complexes (wild type and mutants) were assayed in the presence of increasing concentrations of A769662 through a kinase assay using SAMS peptide. Data were fitted to the equation: Y=Basal+((Activation*Basal-Basal)*X)/(EC50+X). Continuous lines were generated using this equation with the parameters cited in the text. Data represent the average of 3 biological replicates ± SEM. Control AMPK activity corresponds to: WT= 1300 nmol/min/mg; K40A= 1400 nmol/min/mg; K42A= 1400 nmol/min/mg; K40A/K42A= 950 nmol/min/mg.

4.2.3 Dephosphorylation of bacterial AMPK cannot be prevented by A769662, salicylate or AMP

One of the main effects of direct activators of AMPK is to protect Thr172 from dephosphorylation by protein phosphatases. To test if the newly described binding pocket is also the site where some direct activators exert their protective effect against

dephosphorylation, we decided to perform dephosphorylation protection assays in presence or absence of direct activators. The protein phosphatase used for these experiments was PP2Cα and before performing the assay we needed to evaluate for each bacterial protein a concentration of PP2Cα that could decrese Thr172 phosphorylation by around 70%, since the ligands investigated were expected to protect against dephosphorylation but not to prevent it completely. For each bacterial complex we therefore performed a dephosphorylation reaction using serial dilutions of PP2Cα so that we could select a suitable concentration.

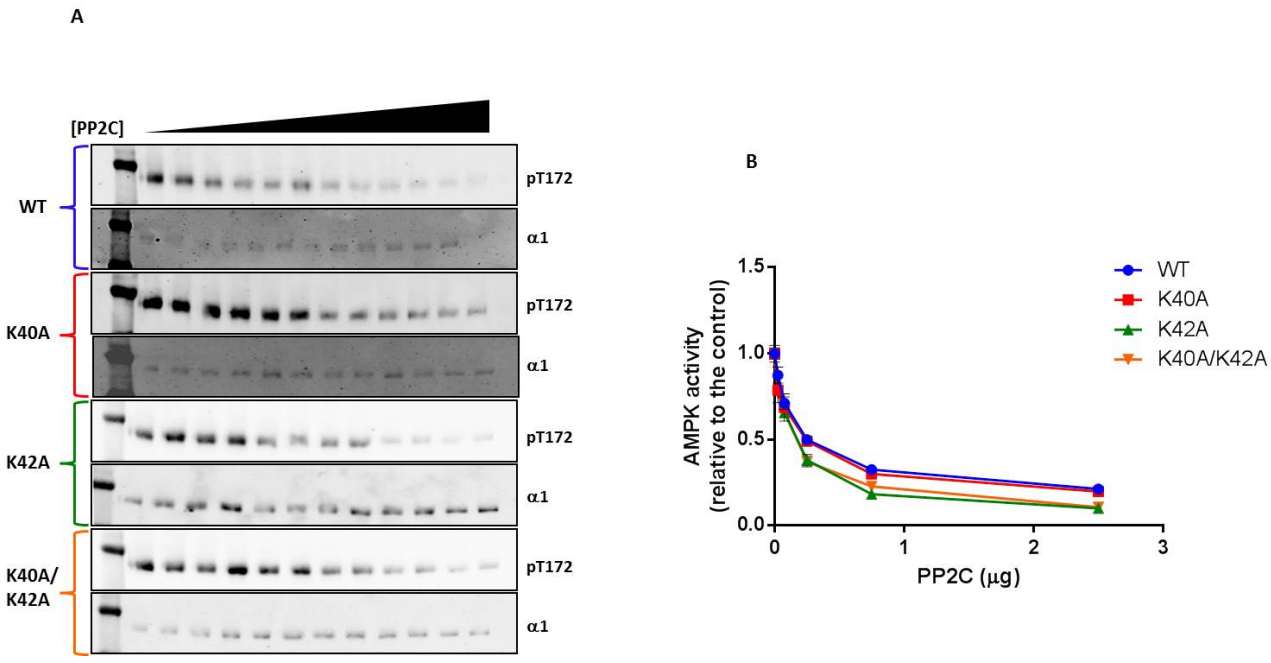

Figure 4.6 Titration with PP2Cα. Wild type and α1β1γ1 mutants were incubated with serial dilutions of PP2Cα. The dephosphorylation reaction was started by the addition of $MgCl_2$ and was stopped by diluting the reaction 16 times with assay buffer on ice. **(A)** Diluted samples were then either used for a Western blot analysis performed with pT172 and α1 antibodies or **(B)** they were assayed in presence of AMARA peptide through a kinase assay. The normalized value of 1 corresponds to: WT= 3000 nmol/min/mg; K40A= 3000 nmol/min/mg; K42A= 7000 nmol/min/mg; K40A/K42A= 6000 nmol/min/mg. The graph represents 3 independent experiments and the error bars represent the SEM.

Figure 4.6 A shows that wild type and K40A mutant required about 0.25 μg of PP2Cα while K42A and the double mutant K40A/K42A needed about 0.15 μg of PP2Cα to yield this extent of dephosphorylation. These findings were confirmed by Western blot analysis shown in Figure 4.6 A, evaluating the phosphorylation of Thr172 and the relative total amount of AMPK-α1 subunit. As a pilot experiment we decided to test whether A769662, AMP and salicylate were able to protect against PP2C-induced dephosphorylation of the wild type bacterial protein. Unfortunately, although there was a modest effect with A769662, almost no protection from dephosphorylation was detected using either AMP or salicylate (Figure 4.7), in contrast to previous observations from our laboratory (Hawley et al, 2012).

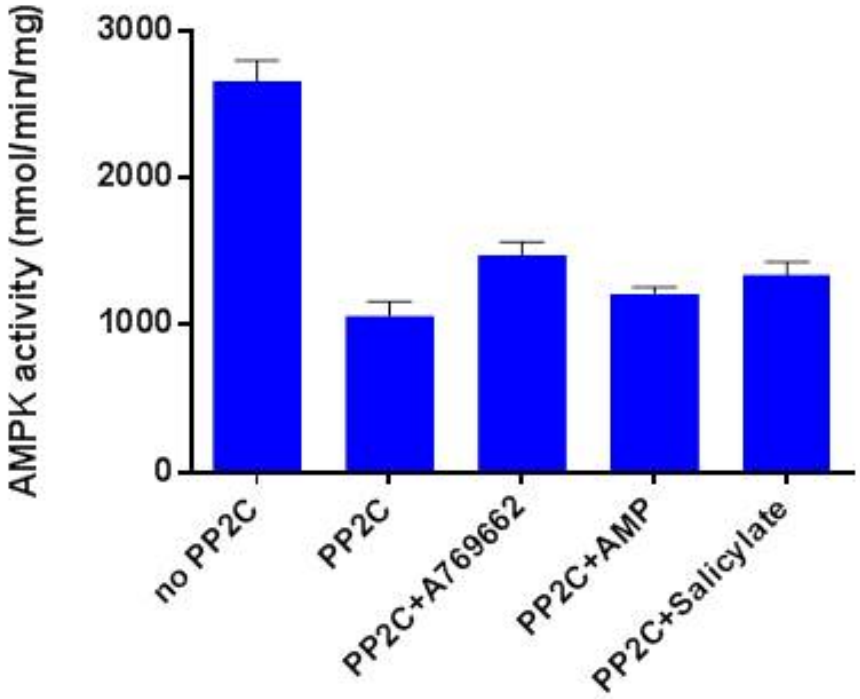

Figure 4.7 Dephosphorylation protection assay. Wild type purified α1β1γ1 was incubated with PP2Cα in the presence of A769662 (1 μM), AMP (200 μM) or salicylate (10mM). A dephosphorylation protection assay was then performed using the AMARA peptide. Data represent the average of 3 biological replicates ± SEM.

For reasons that we do not yet fully understand, previous experience in the laboratory has shown that with some preparations of bacterially expressed heterotrimers it is difficult to detect protection against dephosphorylation by AMPK activators.

4.2.4 Use of unphosphorylated bacterial AMPK to study the effect of direct activators

Given that our current preparations did not allow us to proceed with a full investigation of the novel binding site, and that, unfortunately, there was not enough time to make new preparations of phosphorylated bacterial AMPK, we needed to find an alternative preparation to carry out our studies. Scott et al (2014) recently demonstrated that, despite not being phosphorylated on Thr172, bacterially overexpressed purified heterotrimeric complexes can still respond to both A769662 and AMP. According to their results, the ability of the bacterially expressed complexes to respond to allosteric activators depended on the phosphorylation of Ser108 in the β subunit, and this phosphorylation seemed to be dependent on the temperature at which the bacterial AMPK is expressed. An optimal temperature of 22˚C was suggested, while the standard purification protocol for bacterially expressed AMPK used a temperature of 20˚C or less. In our studies the expression of His-tagged heterotrimeric complexes was induced by the presence of allolactose in the growth medium, followed by a decrease of the incubation temperature, as explained in detail in Materials and Methods. We therefore tested three different temperatures of expression and performed SDS-PAGE, followed by Coomassie Blue-staining, using samples from the pre-induction, induction and post-induction phases. As shown in Figure 4.8 A, the best expression of the α subunit of AMPK could be observed in the post-induction sample, when induction was performed at 25˚C. Once the purification

protocol was optimized, we proceeded with the purification of the wild type and mutated complexes. The four purified complexes could be detected by SDS-PAGE followed by Coomassie Blue-staining (Figure 4.8 B), while the level of their phosphorylation on Ser108, which was comparable, is shown in Figure 4.8 C, top panel.

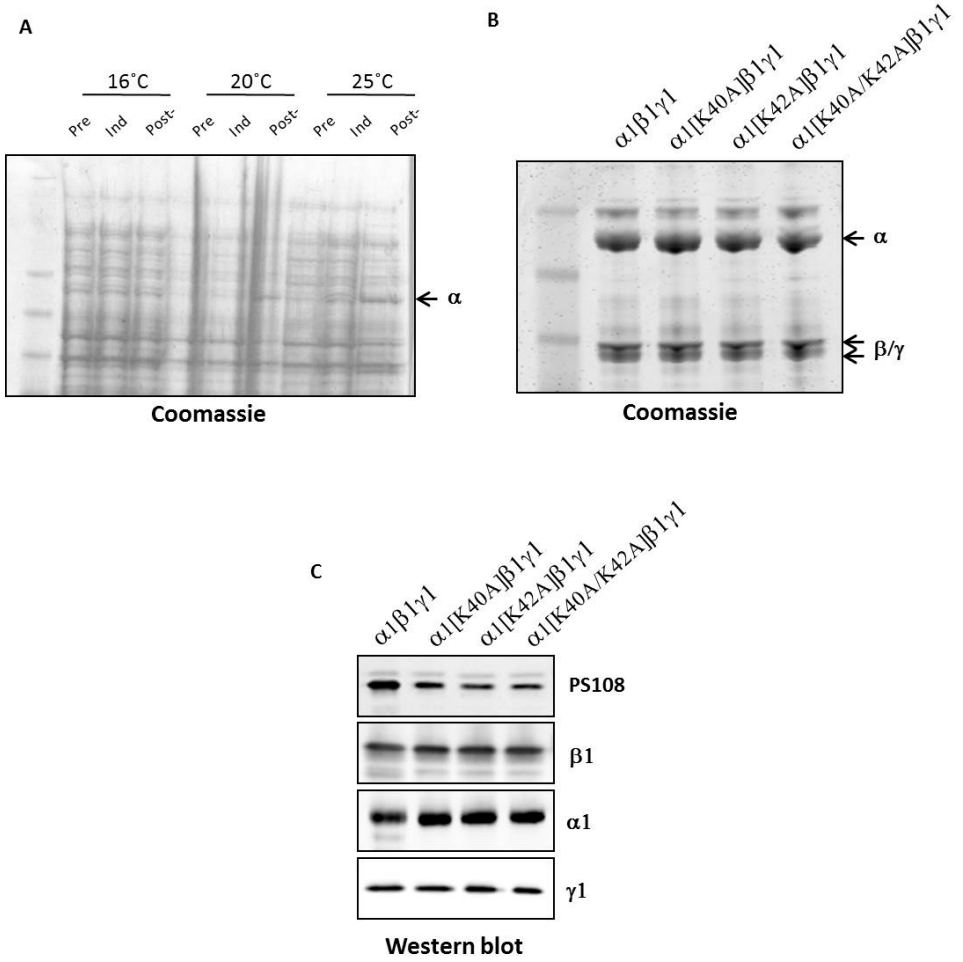

Figure 4.8 Purification and normalization of unphosphorylated α1β1γ1. (A) Three bacterial cultures expressing wild type α1β1γ1 were grown at 37˚C until their OD was 0.6. A sample (*Pre*) was taken from each culture. In each culture the induction of α1β1γ1 expression occurred at different temperatures (16, 20 or 25˚C). A sample from each culture was taken 10 minutes after the induction started (*Ind*). The induction lasted 16 hr and before starting the purification, another sample was taken from each culture (*Post*). All of the collected sample were subject to SDS-PAGE followed by Coomassie Blue staining. Normalization of purified wild type and mutant α1β1γ1 was performed as explained in Figure 4.3, through SDS-PAGE followed by Coomassie Blue staining **(B)** and Western blot analysis with PS108, β1, α1 and γ1 antibodies **(C)**.

We used these unphosphorylated bacterially expressed proteins to investigate their response to four allosteric activators: A769662, 991, MT63-78 and AMP. As expected, the wild type AMPK responded to all the activators, although to different extents.

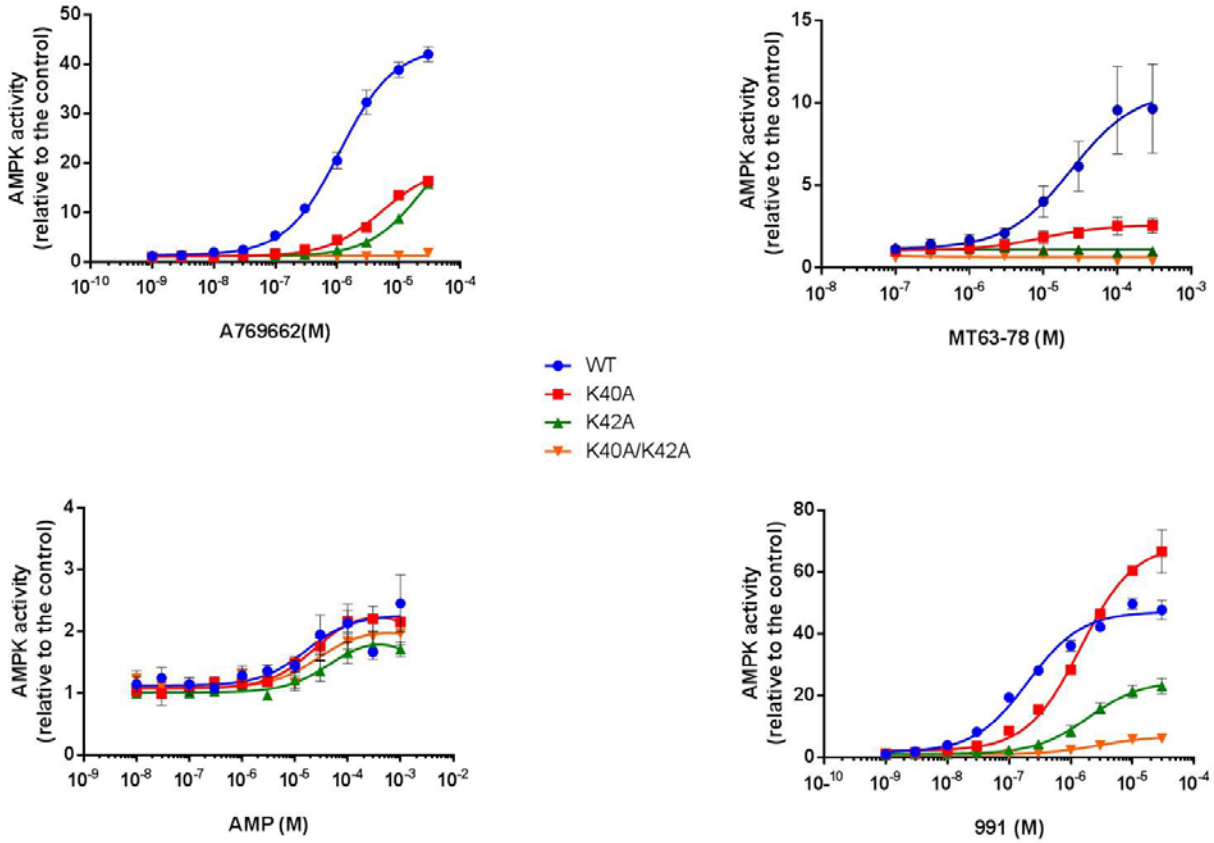

Figure 4.9 K40A/K42A mutant is allosterically activated only by AMP. Purified wild type and mutant α1β1γ1 complexes were tested for their response to increasing concentrations of three direct activators of AMPK and AMP in a standard kinase assay using SAMS peptide. Data obtained with A769662, 991 and MT63-78 were fitted to the equation: Y=Basal+((Activation*Basal-Basal)*X)/(EC50+X), while data obtained with AMP were fitted to the equation Y=Basal+(((Activation*Basal-Basal)*X)/(EC50+X))-(((Activation*Basal)*X)/(IC50+X)). Continuous lines were generated using this equation with the parameters cited in the text. Each graph represents the average of 3 independent experiments ± SEM. Control AMPK values correspond to: WT= 0.3 nmol/min/mg; K40A= 0.2 nmol/min/mg; K42A= 0.1 nmol/min/mg; K40A/K42A= 0.1 nmol/min/mg.

While only a modest 2-fold activation was seen with AMP (EC_{50} = 16 μM), around 30-fold activation could be observed with A769662 (EC_{50} = 1 μM) and 991 (EC_{50} = 0.2 μM), while a 10-fold activation was detected as a response to MT63-78 (EC_{50} = 23 μM). Activation by A769662, 991 and MT63-78 was decreased when the single mutants were tested and was completely abolished when the double mutant was used. In contrast, both the wild type and the mutated complexes responded to increasing concentrations of AMP, confirming once again that the binding site of some direct activators differs from that of AMP, and suggesting that mutations in the newly described binding site only impairs AMPK activation by these direct activators but not by AMP. These results are summarised in Figure 4.9. In their study, Scott et al (2014) also demonstrated that A769662 and AMP can synergistically activate unphosphorylated bacterial AMPK. As shown in Figure 4.10 A, the synergistic effect of A769662 and AMP was significantly impaired in the single mutants and almost completely abolished in the double mutant, similar to what was observed using A769662 alone. However, a small 1.5-fold activation could still be detected in the double mutant protein. This residual activation was entirely dependent on AMP. Figure 4.10 B confirms that all the four complexes responded almost equally to allosteric stimulation by AMP. These data confirm that mutations in the new binding pocket only impair allosteric stimulation of AMPK by some direct activators but not by AMP.

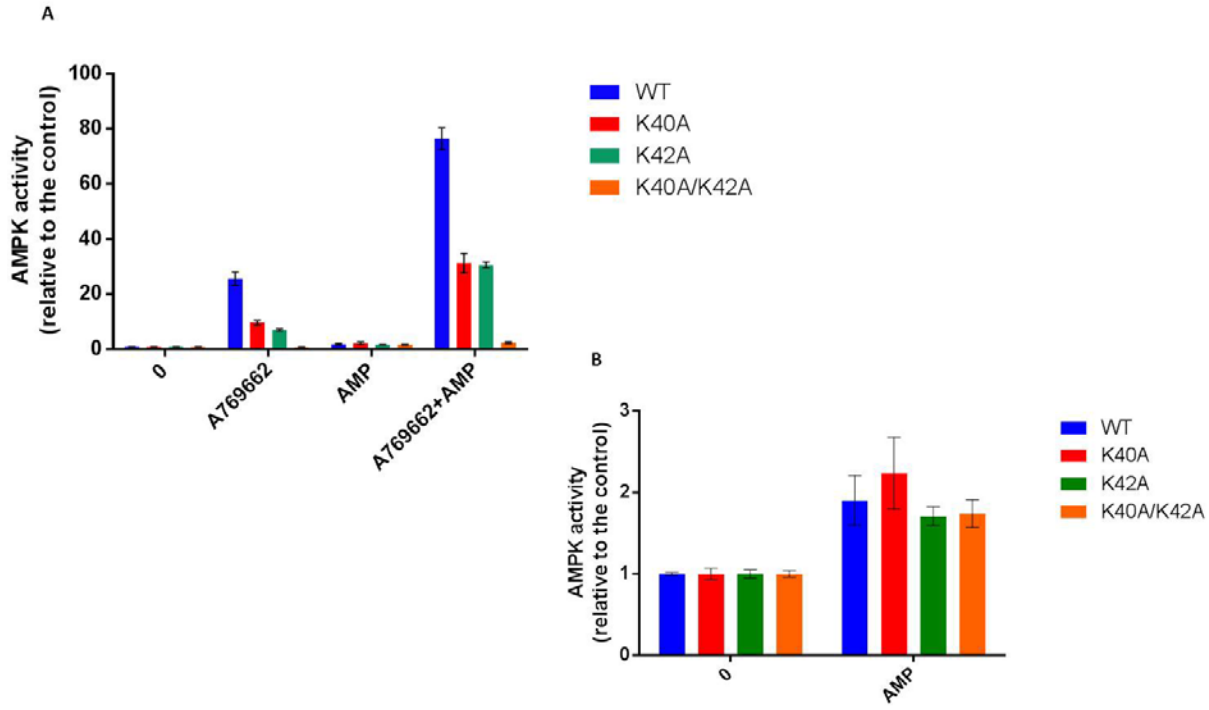

Figure 4.10 Synergistic effect of A769662 and AMP is affected by K40A/K42A mutation. (A) Wild type and mutated α1β1γ1 heterotrimes underwent a kinase assay with SAMS peptide in presence of A769662, AMP or both. **(B)** Plotting of the data from panel A including only the results obtained with AMP alone and using a different scale on the y axis. Data obtained with A769662 were fitted to the equation: Y=Basal+((Activation*Basal-Basal)*X)/(EC50+X), while data obtained with AMP were fitted to the equation Y=Basal+(((Activation*Basal-Basal)*X)/(EC50+X))-(((Activation*Basal)*X)/(IC50+X)). Continuous lines were generated using this equations with the parameters cited in the text. Data represent the average of 3 biological replicates ± SEM. Control AMPK activity corresponds to: WT= 0.5 nmol/min/mg; K40A= 0.4 nmol/min/mg; K42A= 0.2 nmol/min/mg; K40A/K42A= 0.3 nmol/min/mg.

4.2.5 Mutation of Lys40 and Lys42 in α1 and Lys29 and Lys31 in α2 prevents allosteric stimulation of AMPK by direct activators in transfected cells

Experiments performed so far in cell-free assays had confirmed that Lys40 and Lys42 in the α1 subunit were crucial residues for the binding and the effect of some direct activators of AMPK. The CRISPR (Clustered Regularly Interspaced Short Palindromic

Repeats)/Cas9 system is a relatively simple, RNA-programmable method to mediate genome editing in mammalian cells, and can be used to generate gene knockouts in cultured cells. Our laboratory has used this technology to knockout the α1 and α2 subunits of AMPK in HEK-293 cells. In this knockout cell line we reintroduced either the wild type or the mutated α subunits in order to evaluate the effect of direct and indirect activators when the two lysine residues in the novel binding site were mutated. In Figure 4.11 A, α1/α2 knockout HEK-293 cells were transiently transfected with α1 subunit, either wild type or carrying the double mutation of Lys40 and Lys42. Transfected cells were then treated with three different AMPK activators: A769662, salicylate and H_2O_2. From the Western blot analysis shown, it can be seen that all the activators induced phosphorylation of ACC when wild type α1 was transfected. However, in cells transfected with the double mutant of α1, both A769662 and salicylate failed to promote ACC phosphorylation, which was, however, still clearly detectable after the treatment with H_2O_2. The result was confirmed by a kinase assay (Figure 4.11 B) showing that in cells transfected with the double mutant, AMPK could not be activated by A769662 or salicylate while an activation, albeit reduced, was still detectable when cells were treated with H_2O_2. These findings confirmed once more that Lys40 and Lys42 are crucial residues involved in the binding and in the effect of some direct activators of AMPK, and also suggested for the first time that the newly discovered binding pocket is also the site where salicylate exerts its effect. Interestingly, H_2O_2 was still able to stimulate AMPK activation in cells expressing the double mutant. In Chapter 3 of this thesis it was been shown that H_2O_2 activated AMPK primarily by increasing the AMP:ATP ratio, and we have previously shown (Figure 4.9) that AMP maintains its allosteric effect on bacterially expressed AMPK, even when the

double mutation in Lys40 and Lys42 was present. Thus the results obtained in cell-free assays were confirmed by experiment performed in intact cells.

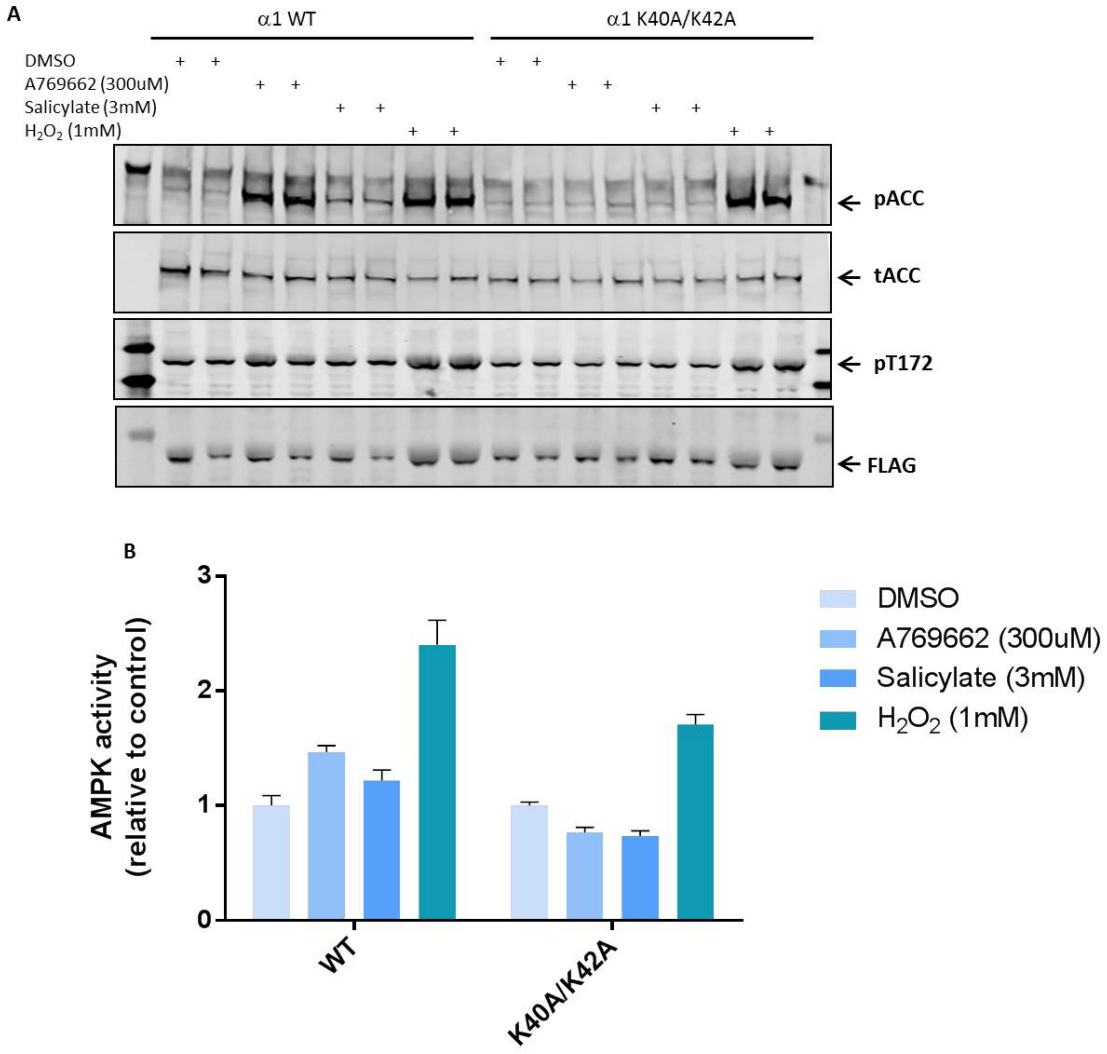

Figure 4.11 The K40A/K42A mutation prevents activation by A769662 and salicylate in transiently transfected cells. HEK-293 cells with a α1/α2 knock out were transiently transfected with either wild type or K40A/K42A α1 subunit carrying a FLAG-tag. Cell were treated with DMSO as negative control, A769662 (300 μM), salicylate (3 mM) or H_2O_2 (1 mM) and then were lysed. **(A)** SDS-PAGE was performed followed by a Western blot analysis with pACC, ACC, pT172 and FLAG antibodies. **(B)** Lysates were immunoprecipitated with FLAG antibody and then subject to a kinase assay using the AMARA peptide. Data represent the average of 3 biological replicates ± SEM. The normalized value of 1 in panel B corresponds to a control AMPK activity of 0.04 nmol/min/mg in both wild type and K40A/K42A transfected cells.

However, when we transfected α1/α2 knockout HEK-293 with the α2 subunit, either wild type or mutated, instead of α1, the outcome was slightly different. As expected, cells transfected with the double mutant form of α2 did not respond to any of the four direct activators used (A769662, salicylate, MT63-78 or 991). However, no response was detected even after treatment with phenformin or H_2O_2, which are known to indirectly activate AMPK by increasing the AMP:ATP ratio. These results are shown in Figure 4.12.

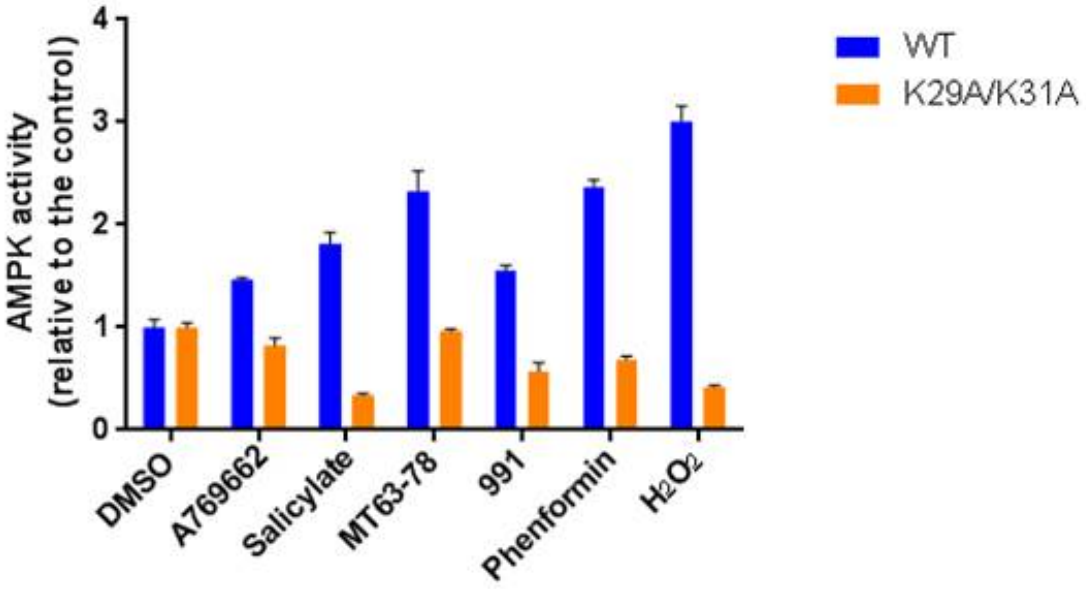

Figure 4.12 AMPK K29A/K31A α2 subunit does not respond to any activator. α1/α2 knockout HEK-293 cells were transiently transfected with FLAG-α2 subunit (wild type or carrying the double mutation) and were treated with a variety of AMPK activators: A769662 (300 μM), salicylate (3 mM), MT63-78 (30 μM), 991 (30 μM), phenformin (3 mM) and H_2O_2 (1 mM). α2-AMPK was immunoprecipitated through an anti-FLAG antibody and its activity assayed by kinase assay using AMARA peptide. The graph represents the average of 3 independent experiments where the error bars represent the SEM. Control AMPK activity corresponds to 0.1 nml/min/mg in both wild type and K29A/K31A transfected cells.

4.2.6 Dephosphorylation protection by direct activators is prevented by mutation of Lys29 and Lys31

The lack of activation of AMPK in response to phenformin or H_2O_2 when Lys29 and Lys31 in the α2 subunit were mutated suggested that this mutation promotes a

change in the AMPK kinase domain such that the effect of AMP was also impaired. We have already shown that the allosteric stimulation by AMP was preserved in AMPK carrying the double mutation (Figure 4.9). However, the effects shown in Figure 4.11 were observed in immunoprecipitates so must have been due to changes in covalent modification (such as phosphorylation) rather than allosteric effects. We therefore considered the possibility that the protection from dephosphorylation induced by AMP was affected by mutation of Lys29 and Lys31. To explore this possibility, we performed dephosphorylation protection assay with PP2Cα, in the presence of absence of AMP, using immunoprecipitated AMPK from transfected cells.

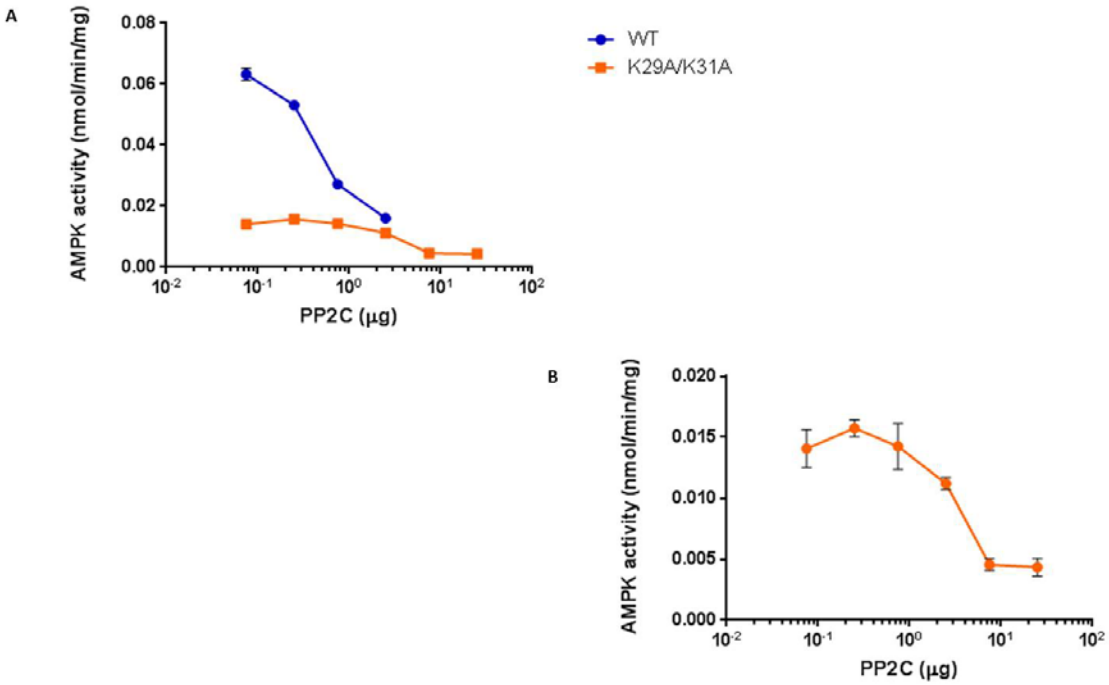

Figure 4.13 Titration of immunoprecipitated AMPK with PP2Cα. (A) α1/α2 knockout HEK-293 cells were transfected with DNAs encoding either the wild type or the mutated α2 subunit. AMPK-α2 was immunoprecipitated by anti-FLAG antibody and the precipitates underwent a 10-minutes dephosphorylation reaction in presence of 5 mM ATP with increasing amount of PP2Cα. After dephosphorylation, samples were assayed for kinase activity using the AMARA peptide. **(B)** Magnification of panel A including only the results with the K29A/K31A mutant, using a different scale on the y axis.

Before performing the experiment, we determined the amount of PP2Cα needed to obtain an appropriate level of dephosphorylation of the immunoprecipitated AMPK. As shown if Figure 4.13 A, almost 1 μg of PP2C was sufficient to dephosphorylate wild type AMPK. However, as shown in Figure 4.13 B, much more phosphatase was needed to dephosphorylate the double mutant; 25 μg of PP2Cα was used to dephosphorylate immunoprecipitated AMPK carrying the K29A/K31A mutation. We then performed the dephosphorylation protection assay using either wild type or mutated immunoprecipitated AMPK in the presence or absence of increasing concentrations of AMP.

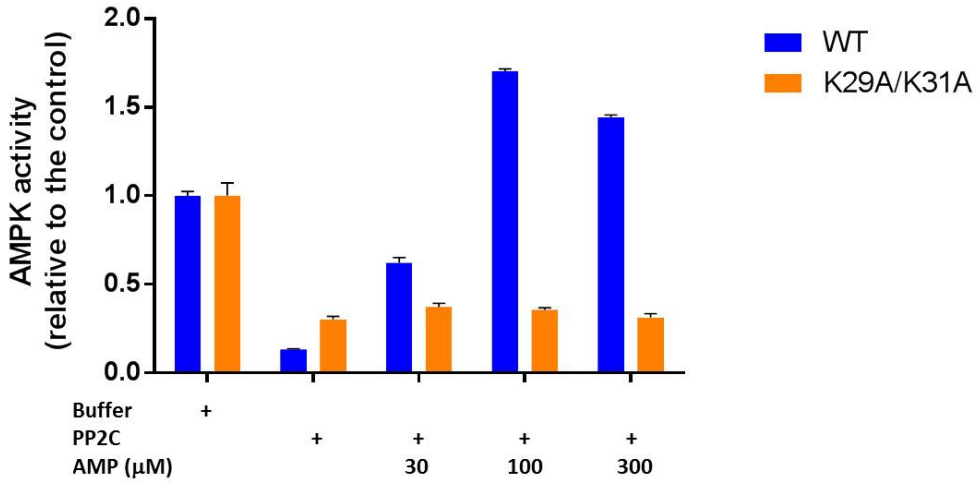

Figure 4.14 AMP does not protect the K29A/K31A mutant against dephosphorylation by PP2Cα. AMPK-α2 (wild type or mutated), was immunoprecipitated from transfected lysate and underwent a dephosphorylation protection assay in the presence of increasing concentrations of AMP. Data were fitted to the equation Y=Basal+(((Activation*Basal-Basal)*X)/(EC50+X))-(((Activation*Basal)*X)/(IC50+X)). Data represent the average of 3 biological replicates ± SEM. Control AMPK activity corresponds to: WT= 0.04 nmol/min/mg; K429/K31A= 0.02 nmol/min/mg.

In immunoprecipitates from cells transfected with wild type α2, 100 μM AMP was able to completely protect against dephosphorylation by PP2Cα, but in immunoprecipitates from cells transfected with the double mutant AMPK activity was insensitive to AMP (Figure 4.14). The same experiment was performed in presence of a single dose of AMP or using four direct activators of AMPK.

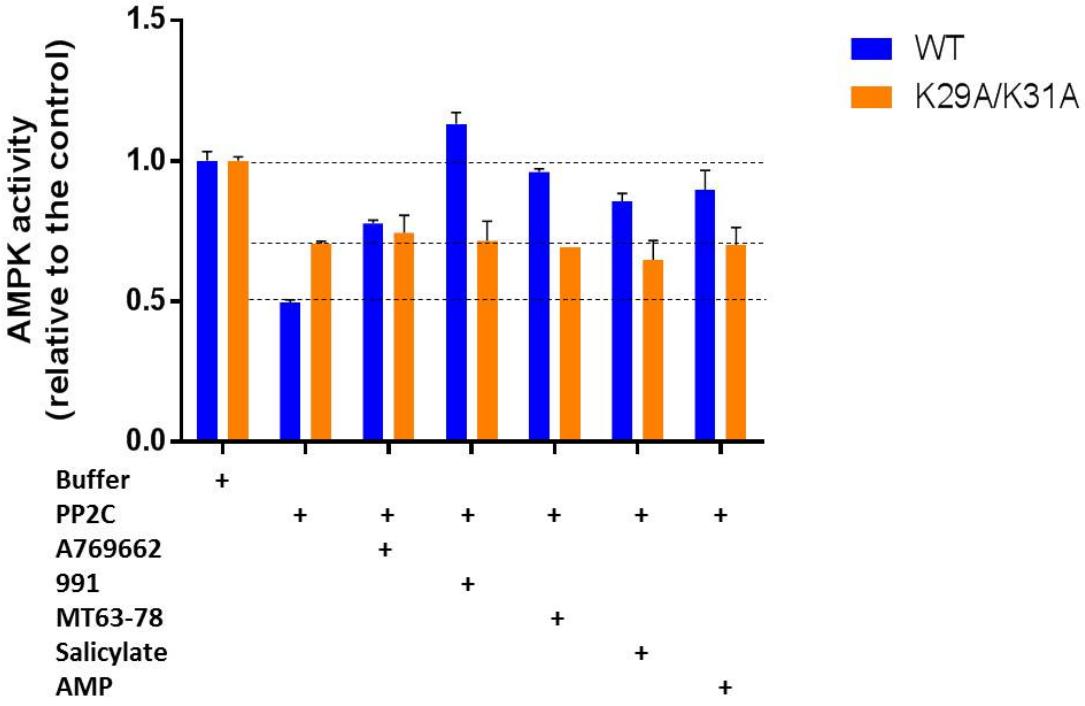

Figure 4.15 AMPK activators cannot protect the K29A/K31A mutant against dephosphorylation. Immunoprecipitated AMPK-α2 from transfected lysates was subject to a dephosphorylation protection assay in presence of different AMPK activators: A769662 (10 μM), 991 (300 nM), MT63-78 (100 μM), salicylate (10 mM) or AMP (200 μM). The graph represents the average of 3 independent experiments with the error bars representing the SEM. The normalized value of 1 corresponds to a basal AMPK activity of 0.07 nmol/min/mg in the wild type transfected cells and of 0.02 nmol/min/mg in K29A/K31A transfected cells.

Figure 4.15 shows that none of the activators used, including AMP, could protect the mutated AMPK against the dephosphorylarion by PP2Cα, showing that mutation of Lys29 and Lys31 in the α2 subunit impairs the dephosphorylation protection effect not

only of direct activators that bind between the α-kinase domain and the β-CBM, but also of AMP, which binds to the γ subunit.These data also suggest that the pocket discovered at the interface between the kinase domain and the β-CBM is the site where all the direct activators known so far bind and activate AMPK.

4.2.7 Basal AMPK activity is severely impaired in stable cell lines expressing a mutated α2 subunit

The next step towards a better understanding of the possible importance of the novel binding site consisted in the establishment of a cell line stably expressing either the wild type or the mutated α2 subunit. To this aim we took advantage of the α1/α2 knockout HEK-293 cell line containing an integrated Flp-recombinase target (FRT) site, which allows the tetracycline-inducible expression of the gene of interest from a specific genomic location (O'Gorman et al, 1991). Once the constructs containing either the wild type or the single and double mutants of α2 were inserted into the FRT site, we tested whether their expression was actually tetracycline-dependent. As shown in Figure 4.15, only samples treated with tetracycline expressed the α2 subunit and the FLAG peptide (all the constructs inserted were tagged with the FLAG peptide).

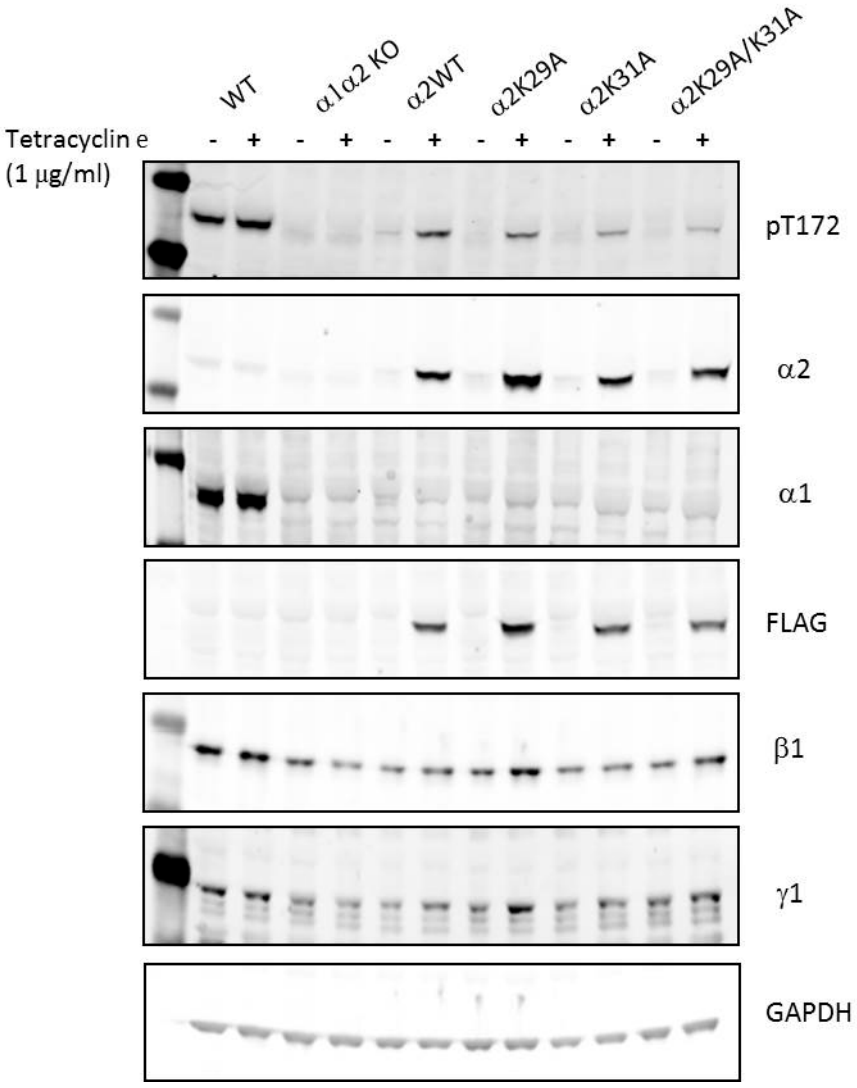

Figure 4.16 Establishment of stable cell lines expressing α2-AMPK either wild type or mutated. α1/α2 knockout HEK-293 cells carrying a FRT-site underwent the protocol to generate stable cells expressing wild-type, K29A, K31A or K29A/K31A double mutant AMPK-α2 (see Materials and Methods). Stable cell lines were treated with or without 1 μg/ml tetracycline for 36 hr to induce gene expression prior to lysis. Lysates underwent SDS-PAGE followed by Western blot analysis with pT172, α2, α1, FLAG, β1, γ1 and GAPDH antibodies. Wild type HEK-293 and α1/α2 knockout HEK-293 were used as positive and negative controls respectively.

Interestingly, as shown in the top panel of Figure 4.16, cells stably expressing the K29A/K31A mutant of AMPK show a dramatic decrease in the basal phosphorylation of Thr172. When we performed a kinase assay comparing the basal activity of wild type and mutated AMPK, we observed that the K29A/K31A mutation resulted in a more

than 5-fold decrease in the basal activity of AMPK (Figure 4.17), even though the expression of the protein was very similar (Figure 4.16).

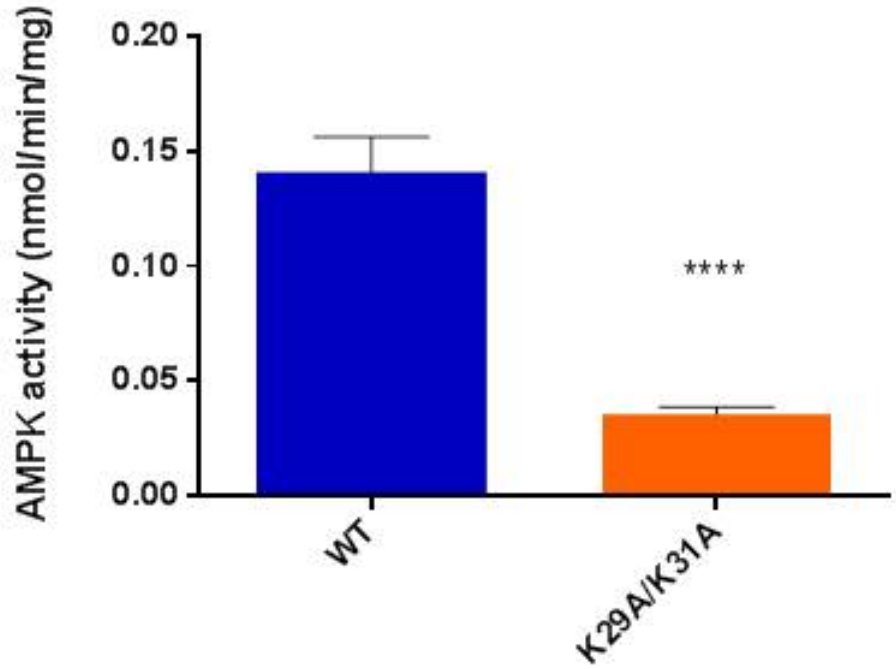

Figure 4.17 The basal activity of the K29A/K31A α2 mutant is lower than the wild type. Stable cell lines expressing either wild type or mutated α2-AMPK were lysed. α2-AMPK was immunoprecipitated by FLAG antibody and its activity assayed by kinase assay using the AMARA peptide. Data represent the average of 3 biological replicates ± SEM. Statistical analysis was performed by unpaired t-test where **** = P≤0.0001.

Our results underlie the importance that the newly discovered binding pocket seems to have in the regulation of AMPK. Unfortunately, our studies with the stable cell lines could not be taken much further. After a few weeks of culturing cells we noticed that, for reasons not yet fully understood, the cell line expressing the double mutant of α2 started to respond to direct activators in the same way as those expressing the wild type subunit. Considering that all our previous studies suggested that this double mutation rendered AMPK almost insensitive to direct activators, we realized that something had changed in the stable cell lines. When we initially tested the α1/α2

knockout HEK-293 cells obtained using the CRISPR/Cas9 methodology, we could detect almost no ACC or Thr172 phosphorylation following the treatment with three different AMPK activators (A769662, H_2O_2 and A23187) as shown in Figure 4.18 A. However, the cells used in this control experiment were grown in media containing a high concentration (25 mM) of glucose. When we performed the control experiment again, we grew cells in a medium containing 5 mM of glucose and tested a larger variety of activators, including the recently discovered activator, SU6656 (Bain et al, 2007). Figure 4.18 B shows that the level of phosphorylation of ACC induced by different treatments, was comparable between the wild type and the α1/α2 knockout HEK-293 cell line. These results suggest that we had not obtained a complete knockout of both the α1 and α2 subunits in our HEK-293 cells. The presence of traces of α1 and α2 subunit could have compromised the generation of the stable cell lines discussed above. In order to continue with this study, it will be necessary to generate the double knockout cell line again, which will be then used to establish stable cell lines expressing either the wild type or the mutated α2 subunit.

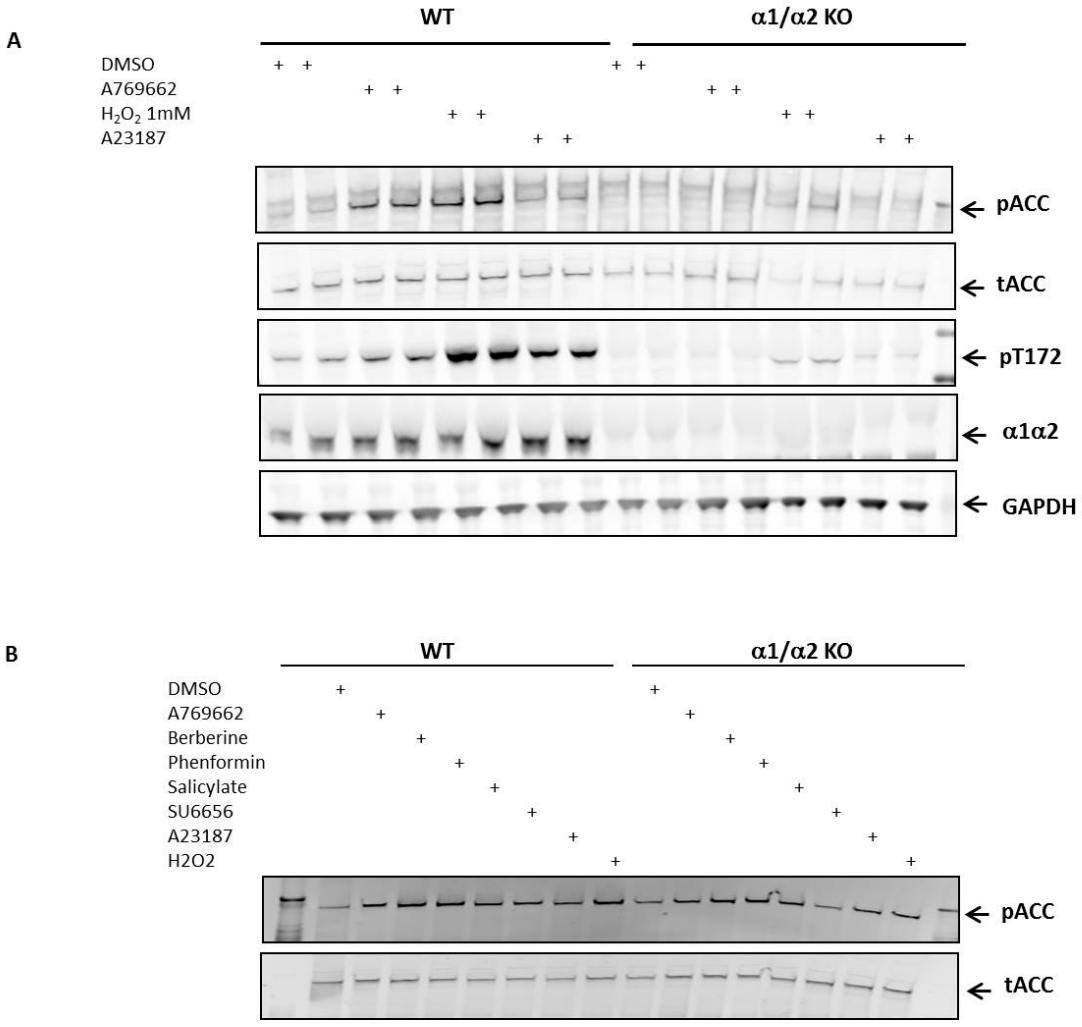

Figure 4.18 Characterization of α1/α2 knockout HEK-293 cells. Standard and α1/α2 knockout HEK-293 were grown in media containing either 25 mM glucose **(A)** or 5 mM glucose **(B)**. They were then treated with DMSO as control, A769662 (300 μM), berberine (3 mM), phenformin (3 mM), salicylate (3 mM), SU6656 (100 μM), A23187 (10 μM) and H_2O_2 (1 mM) and lysed. Western blot analysis was performed with pACC, ACC, pT172, α1α2 and GAPDH antibodies.

4.3 Discussion

The data shown in this chapter contribute to a better characterization of the novel binding pocket located at the interface between the N-lobe of the α-kinase domain and the β-CBM, which was first described by Xiao et al (2013). In their work, Xiao and colleagues identified this pocket as the binding site for both A769662 and the

compound 991, and suggested an important role for Lys29 and Lys31 in the binding pocket. Our data show that mutation of both lysine residues in the α1 subunit of a bacterially expressed heterotrimer (Lys40 and Lys42) almost completely abolished AMPK activation by three of its known direct activators, A769662, 991 and MT63-78 (Figure 4.9). Here we also show for the first time that MT63-78, a newly discovered AMPK activator (Zadra et al, 2014), binds at the same site of A769662 and 991. It has been shown that, in this binding pocket, 991 interacts with hydrophobic residues from both the kinase domain and the β-CBM, mainly through ring-1 and ring-2 of its structure (Xiao et al, 2013). Furthermore, ring-3 and its linkage to ring-2, are involved in some polar interactions. Electrostatic interactions are also formed between Asp88 and N2 of ring-2, and Lys29 and the carboxyl group of ring-3. Moreover, Lys31 interacts with the phosphorylated Ser108 of the β-CBM. The three activators tested share a similar structure, comprising aromatic rings. It is therefore not surprising that mutation of two residues that not only stabilize the structure of the binding pocket itself, but that may also make contact with the activators, severely impairs the response of AMPK to those activators. Unexpectedly, bacterial α1β1γ1 complexes carrying single mutations in these lysine residues, responded in a different way to A769662 depending on the phosphorylation status of Thr172. Thus, K40A and K42A mutant complexes, that had been phosphorylated by CaMKKβ showed the same degree of sensitivity to A769662 as the wild type (Figure 4.5). On the other hand, unphosphorylated K40A and K42A complexes were activated much less by A769662 (Figure 4.9, top left). Although a clear explanation for this result is not yet available, it may be that phosphorylation of Thr172 stabilizes the structure of the binding pocket, counteracting the effects of single mutations. However, mutation of both Lys29 and Lys31 in the α2 subunit of AMPK resulted in such a dramatic change that the presence

or absence of phosphorylation on Thr172 did not make any difference. This could also explain the decreased basal activity of the phosphorylated α1β1γ1 complex carrying the double mutation, observed in Figure 4.4. Interestingly, while the K40A/K42A mutant was not allosterically activated by most of the direct activators tested, it did still respond to AMP (Figure 4.9, bottom left), as also previously found by Xiao et al (2013). However we showed that protection against dephosphorylation of Thr172 was no longer produced by AMP, using AMPK immunoprecipitated from cells transfected with the double mutant (Figure 4.14). These findings can explain why AMPK activators that act mainly by increasing the AMP:ATP ratio, such as phenformin or H_2O_2, also failed to activate AMPK when the K29A/K31A mutant of α2 subunit was expressed in α1/α2 knockout HEK-293 cells (Figure 4.12). As suggested by Li et al (2015), the binding of AMP to the γ subunit promotes the release of the α-AID from the kinase domain by stabilizing the interaction of both the α-AID and α-linker with the γ subunit. This may also cause the catalytic and nucleotide-binding modules to move toward each other, generating a more compact conformation of the complex in which Thr172 is protected from dephosphorylation. If Lys29 and Lys31 are mutated to alanine they would no longer be able to interact with phosphorylated Ser108 on the β-CBM. This may result in a detachment of the β-CBM from the N-lobe of the kinase domain. In this conformation Thr172 might be more exposed to the action of protein phosphatases, thus explaining why both AMP and direct AMPK activators failed to protect the K40A/K42A α2-AMPK from dephosphorylation (Figure 4.15). One region of the heterotrimer that has never been resolved in any of the crystal structures is the connecting peptide that links the β-CBM (part of the catalytic module) and the β-CTD (which forms the core of the nucleotide-binding module). This linker might also be

crucial in the conformational changes that determine accessibility to Thr172, which may therefore be affected by mutations that affect the interaction of the kinase domain and the β-CBM.

Surprisingly, α1-AMPK was still activated by H_2O_2 even when both Lys40 and Lys42 were mutated (Figure 4.11). In Chapter 3 of this thesis we demonstrated that H_2O_2 activates AMPK mainly by increasing AMP:ATP ratio. We also proved that H_2O_2 had a significant role in protecting Thr172 from dephosphorylation, although it is still unclear whether this was a direct effect of H_2O_2 on the phosphatases dephosphorylating AMPK, or an effect mediated by the increase of AMP levels affecting the conformation of AMPK. Therefore the result shown in Figure 4.9 was somewhat unexpected. However, although the reasons for that outcome are still unclear at the present, we have to consider that for all the experiments performed in Chapter 3 we only used cell lines expressing primarily the α1 subunit, and there is the possibility that the mechanism of activation of α2-AMPK by H_2O_2 might show some differences compared to α1-AMPK. It has already been shown that different compounds have different effects according to the α subunit expressed. For instance, it was shown that the AICAR-induced glucose uptake in mouse skeletal muscle is mediated by α2 but not by α1 subunit. The effect was abolished only when α2 subunit was knocked out while knock out of α1 showed no differences compared to the wild type (Jorgensen et al, 2004b). Moreover we had not ruled out a possible direct effect of H_2O_2 on α1 containing complexes, given that RG-γ2 expressing cells still responded to H_2O_2, even if to a lower extent compared to wild type cells. Further investigation will be needed to fully understand the differential response of α1 or α2 double mutants to H_2O_2.

One of the most interesting results of this chapter is shown in Figures 4.16 and 4.17. The stable cell line expressing the K29A/K31A mutant α2 subunit exhibited a basal AMPK phosphorylation and activity which was at least 6 times lower than those in cells expressing the wild type α2. This might suggest that the wild type complex is binding in the cells a natural ligand that affects its net phosphorylation state. If this natural ligand is not able to bind to the double mutant, this would explain the difference in Thr172 phosphorylation and hence activity. If this hypothetical natural ligand exists, its presence at the interface between the β-CBM and the N-lobe of the kinase domain may be crucial for AMPK to maintain its activity, and to regulate all the metabolic processes in which it is involved. However, to date the identity of the possible natural ligand that binds to AMPK at this site remains unclear.

Another possibility which would be worth exploring is that the new binding pocket affects a currently unidentified post-translational modification of AMPK, crucial for its stability and/or activity. The disruption of the binding site (i.e. by mutating Lys29 and Lys31) would prevent the possible interactions between the amino acids of the pocket and the post-translational modification element, thus destabilizing AMPK and/or decreasing its basal activity. This is just a mere speculation, since no evidence so far suggest this scenario. However, it has been demonstrated that AMPK can be ubiquitinated by the laforin-malin complex. The Laforin-malin complex promotes the formation of K63-linked ubiquitin chains in AMPK β subunits (Moreno et al, 2010) and K63-linked polyubiquitin chains are known not to be involved in protein degradation but in other intracellular signalling functions, including the promotion of protein stability (Ikeda & Dikic, 2008). Ubiquitination is only one of the possible post-translational modifications that could be considered and only further studies will allow

us to understand why the binding pocket between the α and β subunits exists, whether it allocates a natural ligand or a post-translational modification, and how it is involved in AMPK regulation.

Chapter 5: Conclusions and future perspectives

5.1 Introduction

In this thesis both canonical and non-canonical mechanisms of activation of AMPK were investigated. AMPK is a crucial metabolic regulator, involved in numerous pathophysiological processes, and the we understand about its regulation, the better we can understand its roles in various human diseases. As well as AMPK, oxidative stress is a process involved in a variety of pathophysiological conditions, raising the question of whether there is an interplay between AMPK and oxidative stress and how that would work. Using different cell lines we studied the effect of H_2O_2, a reactive oxygen species, on AMPK activation, and obtained evidence that it can regulate AMPK through more than one mechanism. Recent works have pointed out the existence of a new binding pocket located at the interface between the N-lobe of the α-kinase domain and the β-CBM of AMPK. It was suggested that this new site was where the synthetic activators of AMPK, A769662 and 991, bound to exert their effect. Using cell-free systems as well as intact cells, we further characterized this novel binding site, showing not only that it is the binding site of four direct activators of AMPK, but also pointing out its possible role in the overall regulation of AMPK. This last chapter discusses the implications of the findings in this thesis and suggests some directions for future research.

5.2 Activation of AMPK by oxidative stress

The work described in Chapter 3 was aimed at understanding the mechanism by which H_2O_2 activates AMPK, in order to clarify the apparent discrepancies between previous work from our laboratory (Hawley et al, 2010) and the study from Zmijewski et al

(2010). While Hawley and colleagues reported that H_2O_2 activates AMPK by increasing the AMP:ATP and/or the ADP:ATP ratios, Zmijewski and co-workers had suggested a direct mechanism of activation, dependent on the oxidation of Cys299 and Cys304 in the α subunit of AMPK. In Chapter 3 of this thesis we showed that, even when we generated H_2O_2 in the cell medium through the addition of glucose oxidase (which is a better model for physiological oxidative stress than addition of a single bolus of H_2O_2 to the medium), cellular ADP:ATP ratio still increased along with AMPK activity suggesting, in contrast to Zmijewski et al (2010), an indirect mechanism of activation. However, our work also revealed that there might be additional mechanisms by which AMPK is activated by H_2O_2. Prolonged incubation with glucose oxidase resulted in a small but significant activation of AMPK in RG cells, which express an AMP/ADP-insensitive mutant of AMPK and are therefore insensitive to changes in cellular nucleotides. This activation was not detected in our previous study (Hawley et al, 2010) because, as we have now shown, a single bolus of added H_2O_2 is rapidly metabolized and has completely disappeared 10-20 minutes after its addition to the medium. This explains why after 60 minutes treatment with H_2O_2 no AMPK activation was detected in RG cells, and only the rapid AMP/ADP independent effect was observed (Hawley et al, 2010). Our data also provided evidence that H_2O_2 activates AMPK by protecting Thr172 from dephosphorylation by protein phosphatases in intact cells. One issue not settled in Chapter 3 is whether this effect of H_2O_2 to protect against dephosphorylation is simply due to the increase in AMP level, caused perhaps by oxidative damage to mitochondria, or whether H_2O_2 has a direct effect on the protein phosphatase(s) that dephosphorylate AMPK. Unfortunately, until the identity of these protein phosphatase(s)is clarified, this question will remain unsolved. Also, we cannot completely rule out the possibility that the small AMP-independent effect of

H_2O_2 on AMPK, observed during prolonged incubation of cells with glucose oxidase, is mediated by a direct oxidation of Cys299 and Cys304 in the α subunit, as suggested by Zmijewski et al (2010). The laboratory is currently in the process of generating HEK-293 cells with a complete double $\alpha1/\alpha2$ knockout. Once these cells are available it would be possible to generate cells stably expressing either a wild type α subunit or an α subunit carrying mutations in those two cysteine residues. This would allow us to investigate whether modification of Cys299 and Cys304 are involved in AMPK activation by H_2O_2.

5.3 Studies of the A769662-binding pocket

The new crystal structure presented by Xiao et al (2013) represented a breakthrough in the field, allowing a better comprehension of both AMPK structure and regulation and in particular identifying the binding site for the synthetic AMPK activator A769662. The work in Chapter 4 of this thesis was aimed to provide a more extensive characterization of the novel binding pocket discovered in the new crystal structure which was located at the interface between the N-lobe of the α-kinase domain and the β-CBM of AMPK. This novel binding site was identified as the site where both A769662 and 991 exerted their effects on AMPK. In our study we could not only confirm this, but we also provided evidence that two other direct activators of AMPK, MT63-78 and salicylate, bind at the same site. We could show that allosteric stimulation of AMPK by A769662, 991 and MT63-78 was almost completely abolished when Lys40 and Lys42 in the $\alpha1$ subunit were mutated into alanine residues. At the same time, allosteric stimulation by AMP, while smaller than that by A769662, was nevertheless unaffected by the double mutation. We also found, rather unexpectedly that the protection against Thr172 dephosphorylation that all the direct activators, including AMP, are

known to exert, was lost when Lys29 and Lys31 were mutated in the α2 subunit. This suggested that the mutation of those two lysine residues might promote a large conformational change in AMPK structure, which could result in Thr172 to be more exposed to the action of protein phosphatases. Lys31 has been reported to make electrostatic interaction with Ser108 of the β-CBM. Further studies should be made to investigate the extent of the conformational modification that might result from mutation of either Lys31 or Ser108. Interestingly we found out that when both Lys29 and Lys31 were mutated in intact cells, the basal activity of AMPK dropped down to almost one sixth of the wild type isoform of the kinase. This outcome suggests a crucial role for the newly discovered binding pocket in AMPK regulation and might indicate the presence of a natural ligand able to bind the wild type, but not the mutant AMPK at that very site. The existence of a natural ligand binding to the same site as A769662 has been an interesting possibility since evidence was obtained in 2007 that A769662 bound at a different site from AMP (Goransson et al, 2007). More studies are now required order to identify this possible ligand. One possible approach might be to generate cell lines expressing either the wild type or the K29A/K31A mutant of α2, it will be possible to immunoprecipitate AMPK and then run mass spectrometry experiments to search for metabolites that might be bound to the wild type complex. Another might be to screen a library of metabolites in cell-free assays using the bacterially expressed, unphosphorylated wild type and K29A/K31A mutant of α2, as described in Chapter 4 of this thesis. Using A769662, the wild type complex was activated >40-fold by A769662, whereas the double mutant was not activated at all. This would therefore provide an excellent assay to screen a library of metabolites. These approaches might open up a new chapter in the story of AMPK, allowing us a better and more complete understanding of AMPK structure and regulation, and

possibly providing new interesting details about its role in different pathophysiological processes.

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